Resolution of enantiomers using cyclodextrins in NMR and HPLC

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in NMR and HPLC

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Summary

The interaction of ß-cyclodextrin with a number of pharmaceutical and other racemates has been investigated in three ways: (i) by HPLC using ß-cyclodextrin-containing eluents, (ii) by HPLC using a ß-cyclodextrin stationary phase (Cyclobond I) and (iii) in NMR experiments. Previously unreported chiral discriminations have been achieved in numerous cases by each technique, namely 24 cases using ß-cyclodextrin eluents, 20 examples using Cyclobond I, and 35 cases in NMR.

The application of ß-cyclodextrin-containing mobile phases in HPLC to the semi-preparative resolution of pharmaceutical racemates has been investigated. An on-line solid-phase extraction recovery system was designed, which allowed recovery of resolved enantiomers free from mobile phase additives. Optical purities of 88% or greater were achieved in the four cases investigated, at throughputs of 1-5mg racemate per hour. The potential applicability of this technique to other chiral separations has been discussed.

The influence of mobile phase composition and stationary phase type on resolution of enantiomers using ß-cyclodextrin eluents have been investigated with the aim of facilitating the optimisation of both analytical and semi-preparative chiral separations.

Correlations between observed chiral discriminations in the three techniques were sought, with a view to using NMR or Cyclobond experiments for the rapid evaluation of whether a given racemate would be likely to be resolvable using ß-cyclodextrin-containing HPLC eluents. Potentially useful empirical correlations were found, although the theoretical basis for these was not strong.

NMR experiments were used to investigate the stability and structure of ß-cyclodextrin complexes. A theory was developed which allowed in some cases the determination of the stability of the diastereomeric complexes from measurements on a racemate.

Contents

Chapter 1

Introduction

1.1 Chirality and pharmaceutical development

In recent years, it has become increasingly clear that chirality has important implications in pharmaceutical development. Hitherto, many chiral drug compounds have been developed and marketed as racemates. However, recent advances in chiral synthesis and chromatographic resolution of enantiomers have opened up the possibility of single enantiomer pharmaceutical products. Research into the pharmacokinetic and pharmacodynamic properties of enantiomers has shown that there may be major advantages in terms of drug efficacy and safety in following this course. Regulatory authorities have indicated that legislation may soon compel investigation of individual enantiomers in any new drug submission.

1.1.1 Fundamentals of optical isomerism

The phenomenon of optical isomerism was first discovered by Pasteur (Pasteur (1948)). He found that on crystallisation of sodium and ammonium salts of synthetic tartaric acid, two types of crystal were formed which were non-superimposable mirror-images of each other. Mechanical separation of the crystals enabled the examination of their properties. Their melting points, refractive indices, solubilities, *etc.* were found to be identical. They were found to rotate the plane of polarised light to the same degree, but in opposite directions. The starting material possessed no such optical rotation, which Pasteur correctly attributed to the "cancelling out" of contributions of the two forms. Natural tartaric acid was found to possess the same optical rotatory power as one of the two crystal forms produced from synthetic material. Pasteur noted that this property frequently distinguished natural from synthetic materials. Pasteur proposed that the asymmetry of the crystals was derived from asymmetry of the molecules that make them up. As ideas of chemical structure were further developed, this hypothesis could be placed on a firmer footing. Thus, the idea of the asymmetric carbon atom (or "chiral centre"), *i.e.* one with four different groups attached, was

first proposed by van't Hoff (1874) and Le Bel (1874). Such a carbon atom is shown in Figure 1.1.

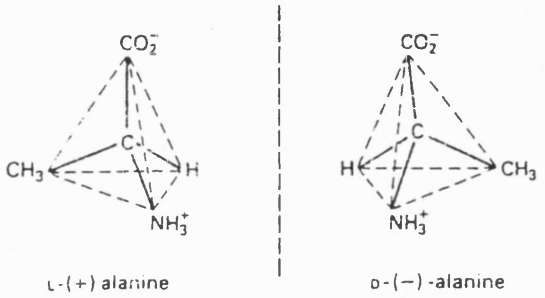


Figure 1.1 The enantiomers of alanine, showing the four different groups attached to the chiral centre (from Mason (1989)).

A number of forms of nomenclature have been devised to describe optically active compounds. Species which rotate the plane of polarised light anti-clockwise are designated "-", while those that cause clockwise rotation are designated "+". It is not possible, however, to relate measured optical rotation to the spatial arrangement of atoms about the chiral centre. The latter may be described by the Cahn-Ingold-Prelog convention, which classifies a chiral centre as R- or S-. In this convention, the groups attached to the chiral centre are classified in order of increasing atomic number, and the designation made on the basis of the order of the groups when the molecule is viewed from opposite the group with lowest atomic number. Absolute configurations may be detemined by "anomalous dispersion" in X-ray crystallography. This was first achieved for the sodium rubidium salt of (+)-tartaric acid (Bijvoet *et al* (1949)).

Many compounds possess more than one asymmetric carbon atom. In general, *n* chiral centres give rise to 2^n optical isomers. Thus, for a molecule with two chiral centres, four isomers may arise, designated as RR, SS, RS, and SR. The RR and SS forms (and similarly the RS and SR) are mirror images but the RR and RS forms (and all other pairs related by

inversion of just one of the chiral centres) are not enantiomeric and may differ in their physical properties. Such pairs of isomers are known as diastereomers.

1.1.2 Pharmacokinetic implications of chirality

As Pasteur observed (Pasteur (1901)), most biological molecules (notably sugars, peptides and proteins) are homochiral, *i.e.* only one enantiomer is present in natural systems. Their interactions with drug enantiomers are therefore frequently stereospecific. If a drug compound is administered as a racemate, differences in both the pharmacokinetics and pharmacodynamics of the two enantiomers may occur.

The pharmacokinetics of a drug are determined by four processes - adsorption, distribution, metabolism and excretion. Stereoselectivity may arise at any of these stages, and will lead to changes in the concentration ratio of two drug enantiomers with time and administration route. This in turn leads to differences in the exposure of physiological systems to the actions of the two enantiomers. Patient-to-patient variation will also arise, influenced by factors such as plasma protein concentrations, metabolic dysfunction due to disease or genetic factors, interactions with other drugs, and age.

In general, processes that involve passive diffusion, such as adsoption in the gut, are not stereoselective. Active transport processes, such as protein binding, passage across membranes and renal tubular secretion, are frequently stereoselective, however.

It is well established that the binding of many drugs to the main plasma proteins - serum albumin and α_1-acid glycoprotein - is enantioselective (Muller (1988)). Thus drugs such as ketoprofen (Rendic *et al* (1980)) and warfarin (Brunner and Muller (1985)) have been shown to bind stereoselectively to albumin; propanolol (Brunner and Nuller (1985)) and disopyramide (Lima *et al* (1984)) (amongst others) bind stereoselectively to α_1-acid glycoprotein. The binding constants of the enantiomers of these drugs to proteins have been shown to differ by

factors of up to 3 (Rendic *et al* (1980)). The resulting differences in free drug enantiomer concentrations in plasma have been widely reported (Muller (1988)).

The metabolism of two enantiomers may also differ significantly. The pharmacokinetics of propanolol enantiomers differ markedly, with the S-isomer exhibiting significantly higher bioavailability (Lindner (1989)). Walle *et al* (1988) have shown that propanolol metabolism is significantly stereoselective. First-pass metabolic ring oxidation occurs to a greater extent (about 2:1) for the R-(+)- isomer, and the degree of first pass metabolism in a given patient largely determines the relative plasma levels of the two enantiomers (von Bahr *et al* (1982)), with considerable inter-individual variation seen. The 2-arylpropionic acids, a widely-prescribed series of non-steroidal anti-inflammatory agents, provide an interesting case. Here metabolic inversion of the inactive R isomer to the active S form has been shown to occur during absorption from the gastro-intestinal tract (Hutt and Caldwell (1983)). Variations in the rate of this metabolic route, which are particularly marked in elderly patients, lead to significant inter-individual differences in plasma levels of the active isomer. It has been suggested that this factor contributed to the toxicity problems which led to the withdrawal of benoxaprofen (Opren) from the market in 1982 (DeCamp (1989)).

1.1.3 Pharmacodynamic implications of chirality

Pharmacodynamic differences between drug enantiomers have been shown to arise in a number of cases. Such differences arise largely because of differences in binding at potential receptor sites. Several possibilities have been identified (Krstulovic (1989), Powell *et al* (1988)):

(1) pharmacological activity resides entirely in one enantiomer;

(2) the enantiomers have identical activity;

(3) the enantiomers have qualitatively similar activities but differ quantitatively in their intrinsic activities;

(4) the enantiomers have qualitatively different activities and act independently;

(5) the enantiomers have opposite activities (possibly due to competitive antagonism of one for the other);

(6) one enantiomer gives rise to the desired therapeutic effect, while the other gives rise to unwanted (side) effects;

(7) one enantiomer inhibits the side-effects due to the other.

In cases where these can be clearly distinguished, the enantiomer giving rise to the desired effect is known as the eutomer, while the other enantiomer is known as the distomer. The ratio of the activity of the two is known as the eudismic ratio (Lehmann *et al* (1976)). Because stereoselectivity is dependent on the specificity of the drug-receptor interaction, high eutomer activity is frequently associated with low distomer activity, and hence a high eudismic ratio. This generalisation is known as "Pfeiffer's rule", and holds when the portion of the molecule interacting with a receptor contains the chiral centre (Ariens (1983)).

Propanolol (marketed as Inderal) is the most widely used ß-adrenoceptor blocking drug, and is administered as a racemate. It also has membrane stabilising and anaesthetic effects. As has already been pointed out, the two enantiomers of this compound differ in their binding to plasma proteins and in their metabolism. The S:R ratio in plasma after administration of the racemate therefore depends on the administration route (Olanoff *et al* (1984)). The ß-blocking activity of the S enantiomer is 100 times higher than that of the R isomer in animal studies, but the anaesthetic and other effects of the two enantiomers are the same (Silber *et al* (1982)) . Therefore, in treatment of some conditions (such as angina) the S enantiomer is more effective than the racemate (Wilson *et al* (1969)), while in others (such as migraine) there is little stereoselectivity in the dose-effect relationships (Stensrud and Sjasstad (1976))

Warfarin is used as an anti-clotting agent. In humans, S-warfarin is a five times more potent

anti-coagulant than the R- isomer, but the S-isomer is eliminated 2-5 times more rapidly. The overall activities of the enantiomers on administering the racemate are therefore similar. However, interactions between warfarin and co-adminstered drugs such as phenylbutazone have also been shown to be stereoselective (O'Reilly *et al* (1980)). The interpretation of clinical data has been more problematic than would be the case if warfarin were to be administered as a single enantiomer.

Thalidomide represents perhaps the greatest disaster in the history of modern pharmaceuticals. It was prescribed for its sedative and anti-nausea properties, often to pregnant women suffering from morning sickness. It rapidly became clear that thalidomide was severely teratogenic, and that the development of this drug had been seriously flawed. A number of authors have suggested that the racemic nature of marketed thalidomide had a part to play in the tragedy. Simonyi (1984) has noted that the teratogenicity in rabbits of racemic thalidomide was significantly greater than that of either enantiomer. Blaschke *et al* (1979) have reported the results of studies where teratogenicity was concentrated in one isomer only. DeCamp (1989) has recently reviewed all the available evidence. He concluded that there is strong, but not conclusive, evidence that thalidomide chirality may have an influence on its side-effects. When thalidomide was marketed, chiral resolution methods were much less well developed. The implication is that the thalidomide tragedy might have been avoided if its enantiomers had been available for separate investigation.

Brewster *et al* (presented at the *7th. Int. Conf. Prostaglandins and related cpds.*, Florence, Italy (1990)) investigated a number of 1,3-dioxane racemates possessing a pyridyl substituent with respect to their thromboxane TXA_2 antagonism and synthase inhibition properties. Compounds exhibiting such activity might have therapeutic value in several conditions by inhibiting the vaso-constrictory and platelet-aggregatory properties of TXA_2 while enhancing the biosynthesis of the anti-aggregatory and vasodilatory species prostacyclin. One compound in particular (TA5 on p.55) was found to combine strong TXA_2 receptor

antagonism and synthase inhibition. Investigation of the properties of the individual enantiomers of TA5 showed that almost all the TXA_2 antagonism property of the racemate arose from the (-) isomer, while the TXA_2 synthase inhibition was due mainly to the (+) isomer. The latter effect was attributed to enantioselective absorption of TA5 into platelets. Clearly, in this case the use of a drug preparation containing both isomers (although not necessarily in racemic mixture) might be advantageous.

1.1.4 Regulatory implications of drug stereoisomerism.

It is clear from the above examples that significant pharmacological differences between drug enantiomers may be the rule rather than the exception. It has been argued that significant advantages in terms of efficacy and safety might accrue from the development of single enantiomer drugs. Thus, Caldwell (presented at *2nd. Int. Symp. Chiral Sepns.*, Guildford, UK (1989)) has pointed out that single enantiomer drugs would

(1) simplify dose-response relationships

(2) reduce inter-individual variation

(3) facilitate extrapolation of animal data and

(4) reduce side-effects in many cases.

Simonyi (1984) has pointed out a number of cases where it might be particularly beneficial to reduce overall dose by administration of single enantiomers. These are for elderly patients, where metabolism may be slow and co-administration of several drugs is common; pregnant women, where protection of the foetus requires extra care in drug administration; and patients with impaired renal function, where accumulation of drug and metabolites may occur. Drayer (1988) has argued that single enantiomer drugs would enhance the capabilities of therapeutic drug monitoring in attempting to relate dosing more closely to the requirements of the individual patient.

Most chiral drugs are currently marketed as racemates. Thus, in a 1982 survey of drugs

marketed in Germany (Ariens and Wuis (1987)), nearly all synthetic chiral drugs (representing about 25% of all drugs in the survey) were marketed as racemates. Almost all the single enantiomer drugs (also representing about 25% of the survey) were natural or semi-synthetic. This illustrates the extreme reluctance of the pharmaceutical industry until recently to invest the extra time and money required to synthesise homochiral drugs (or carry out resolution of racemates).

The case against the marketing of racemic drugs has been put most forcefully by Ariens (1984, 1987). He has argued that the presence of an isomeric impurity at 50% level would not be deemed acceptable if the impurity was anything other than enantiomeric, and that the different pharmacological properties of enantiomers should mean that they are treated as separate chemical entities. In particular, the practice of carrying out pharmacokinetic studies on the racemate without differentiating the enantiomers has been strongly criticised.

The response of the pharmaceutical industry to this criticism has often been that the methods for synthesising or resolving optically pure pharmaceuticals are not well enough developed for their application on a commercial scale. In the last ten years, however, these techniques have advanced so far that this argument is no longer tenable.

In recent publications, it has become clear that the regulatory authorities in the UK and the USA will require full information on the properties of the individual isomers of a racemic new drug, and some justification for the marketing of the racemate as opposed to either enantiomer. (DeCamp (1989); Bridges, J.W., presented at *2nd. Int. Symp. Chiral Sepns.*, Guildford, UK (1989)). At the moment, drug registration legislation does not specifically cover the implications of chirality, but the requirements for full characterisation of the drug substance can be interpreted to include optical purity.

Whether the drug products of the future are marketed as single enantiomers or as

racemates, it is clear that approaches to the production of pure enantiomers, if only in comparatively small quantities for pharmacological evaluation, will continue to occupy the minds of researchers. Analytical methodology for optical purity analysis and chiral analysis in biofluids, which has largely come to the fore only during the last ten years, will be needed in the development of all chiral drugs.

1.1.5 Approaches to the development of single enantiomer drugs

The production of pure enantiomers may be achieved either by chiral synthesis, or by resolution of racemic product. Both approaches have been the subject of intensive research in the pharmaceutical sector.

There are two main ways of synthesising homochiral products without the need for resolution of enantiomers (Scott (1988)). In asymmetric synthesis, chirality is introduced by reaction at a prochiral site (such as C=C or C=O), often using a chiral catalyst or enzyme. A typical reaction of this type is hydrogenation of an alkene over a chiral rhodium-phosphine catalyst, as used in the synthesis of L-DOPA (shown in Figure 1.2)

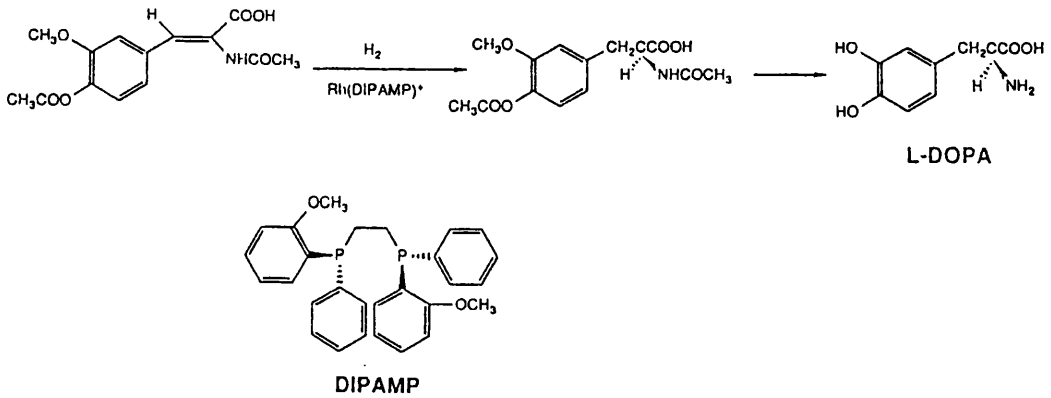


Figure 1.2. Industrial asymmetric synthesis of L-DOPA (from Scott (1988)).

The use of a homochiral starting material may also lead to a homochiral product. For this purpose, a variety of natural products, including sugars and amino acids are frequently employed. This approach is exemplified by the synthetic route to phenothiazines, illustrated in Figure 1.3.

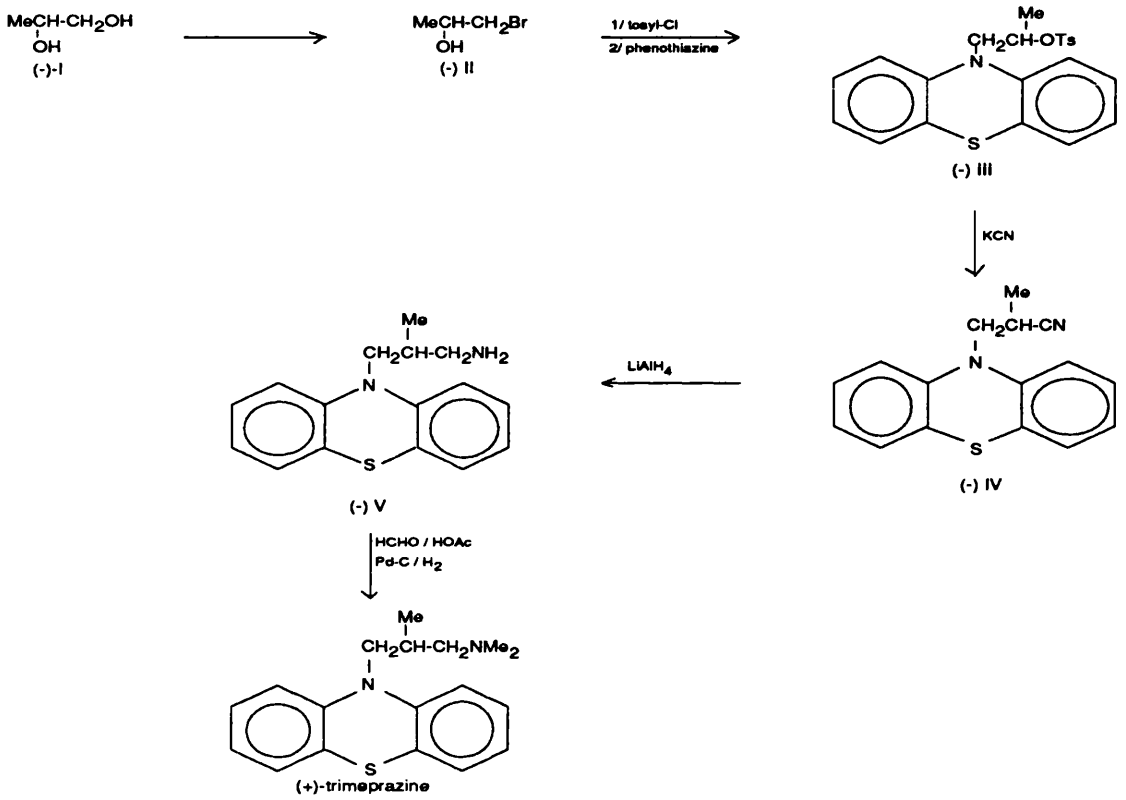


Figure 1.3. Trimeprazine synthesis from chiral starting material (French Patent (1958), US Patent (1958))

If a synthetic route using one of the above approaches is not feasible, resolution of racemic target or synthetic intermediates may be possible. There a number of classical methods of achieving this. The method Pasteur used to initially resolve tartaric acid enantiomers, *i.e.* manual separation of enantiomeric crystals (Drayer (1988)) is both laborious and limited in application, since most racemates do not crystallise to form enantiomeric crystals. However, Pasteur also recognised that the formation of diasteromers by reaction of the racemate with a homochiral reagent would allow the separation of enantiomers. Thus, he resolved tartaric

acid by salt formation with quinine followed by fractional crystallisation. A variation on this approach is kinetic resolution, where one enantiomer of a racemate reacts selectively with chiral reagent (often an enzyme). Pasteur himself used a similar method to resolve tartaric acid enantiomers, utilising the selective metabolism of the d-tartrate by yeast. Both these approaches have since been extended. Diastereomers may be separated either by crystallisation (the classical approach), or chromatographically - an approach that has increased in importance in recent years and is discussed in detail below.

An inherent drawback of resolution over chiral synthesis is that 50% of the material is wasted, if only one enantiomer is required. This problem may be overcome by the racemisation of the unwanted enantiomer and further resolution. While chiral synthesis is usually the method of choice for process-scale production of pure enantiomers, the complexity of developing such syntheses is such that resolution is often employed at earlier stages of drug development, where time is more critical than cost, to produce smaller quantities of homochiral materials. Chromatographic resolution techniques are also widely employed in analytical applications, where the measurement of the concentrations of two enantiomers is the aim, and not their isolation.

1.2 Chromatographic chiral separations

In the last fifteen or so years, chromatographic techniques, and particularly high-performance liquid chromatography (HPLC) have become predominant in pharmaceutical analysis and separations, owing to their high speed, efficiency and ease of automation. This has been particularly true in the separation and analysis of enantiomers, where selectivities are often low, and high separation efficiencies are therefore critical. Chromatographic separation of enantiomers may be effected either by derivatisation and separation of the resulting diastereomers, or by incorporation of a chiral species into the stationary or mobile phase. The development of chiral chromatography has been rapid in recent years, and a plethora of chiral derivatising reagents, stationary phases, and mobile phase additives are now in use.

1.2.1 Indirect chiral separations

Chromatographic separation of diastereomers, formed by reaction of a pair of enantiomers with an optically pure chiral reagent, is termed "indirect" chiral separation, since the chromatographic process itself is not chiral. Thus, in this approach, conventional mobile and stationary phases are used. It is similar in principle to the "classical" resolution methods involving fractional crystallisation of diastereomers discussed above, but has the advantage of greater efficiency. Thus, a separation that would involve many recrystallisations to achieve high optical purity may be achieved in one step on an efficient modern chromatographic column.

A wide variety of chiral derivatisation reactions have been devised. 45 reagents are listed in a recent review (Ahnoff and Einarsson (1989)). Typical reactions are the formation of amides, carbamates and ureas from amines; the formation of esters, carbonates, and carbamates from hydroxyl groups; and the formation of esters and amides from carboxylic acid groups. This approach has found wide pharmaceutical application, such as in the

separation of enantiomers of ß-blockers (Wilson and Walle (1984)), and 2-arylpropionic acids (Hutt *et al* (1986)). The reagents employed are often cheap and readily available, and the analytical methods are often more robust than those using chiral stationary phases.

As a number of reviewers have pointed out, there are distinct drawbacks to the chiral derivatisation approach (Lindner (1988), Karnes and Sarkar (1987), Krstulovic (1989)). The optical purity of the reagent is critical. For example, a 2% optical impurity in an (R)- reagent would lead to the formation of 98% (RR) product and 2% (RS) product on reaction with (R) analyte. The (RS) product would be enantiomeric with (SR), formed by reaction of the (R) reagent with (S) analyte. Since these isomers would be indistinguishable in an achiral chromatography system, erroneous results would be obtained in any optical purity assay of the analyte. It is therefore important to check the optical purity of a reagent before use. Reaction conditions must be chosen so as to avoid racemisation, and reactions should proceed to completion in order to avoid errors due to differential reaction rates for the two analyte enantiomers. In preparative applications, the reversal of the derivatisation to recover the separated enantiomers may be problematic. It has been suggested, therefore, that direct chiral chromatography, involving transient diastereomeric interactions in stationary or mobile phase, may be preferable where possible.

1.2.2 Principles of chromatographic chiral recognition.

The possibility of using chromatographic techniques to resolve racemates was first recognised by Wilstatter (1904)) who proposed enantioselective adsorption onto wool as a means of resolving racemic dyestuffs. Other early chiral separations used other naturally-occurring chiral materials such as lactose, quartz and paper.

The first attempt to rationalise such separations was made by Dalgliesh (1952). He investigated the resolution of various amino acid enantiomers by paper chromatography, and

proposed what has become known as the "three-point attachment" rule to account for the observed variation of enantioselectivity with structure. This generalisation is derived from the Cahn-Ingold-Prelog convention, whereby the positions of three of the four bonds around a tetrahedral carbon must be known in order to specify the configuration as R or S. In the case of chiral recognition, Dalgliesh proposed that at least one of the analyte enantiomers must be capable of at least three simultaneous interactions with the chiral selector, and that at least one of these interactions should be different for the two enantiomers, for chiral recognition to occur. This is illustrated in Figure 1.4, where one enantiomer is capable of three interactions with the selector while the other is capable of only two.

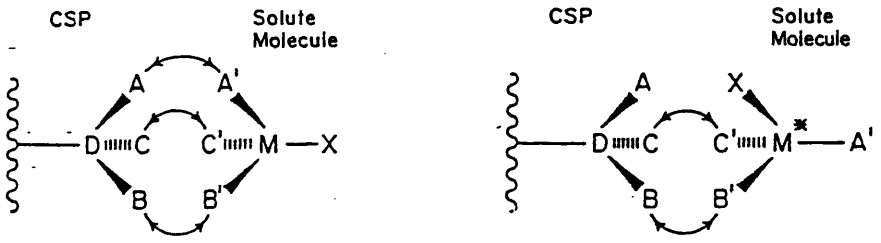


Figure 1.4. Interaction of enantiomeric analytes with a chiral selector, showing the "three-point" interaction required for chiral recognition (from Pirkle and Hamper (1987))

Pirkle has developed the three-point rule further. (Pirkle and Hamper (1987), Pirkle and Pochapsky (1989)). He has pointed out that the analyte-selector interactions may be attractive (*e.g.* hydrogen bonds, electrostatic interactions, dipole-dipole forces, pi-acid-pi-base interactions, hydrophobic effects) or repulsive (*e.g.* steric interactions). If the stereochemically-dependent interaction is attractive, *i.e.* free-energy lowering, the analyte-selector complex of that enantiomer capable of all three interactions will be stronger. If the

stereoselective interaction is repulsive (*i.e.* energetically unfavourable), the analyte-selector complex with least contribution from that interaction will be stronger.

Chromatographic retention order may thus be rationalised. If the chiral selector is bound to or coated on the stationary phase, the analyte enantiomer forming the strongest analyte-selector complex will be most retained. If the chiral selector is added to the mobile phase, the analyte forming the strongest analyte-selector complex will be most prevalent in the mobile phase, and therefore will elute most rapidly (*i.e.* will be least retained).

The above arguments apply only to "point" interactions. Some interactions (such as pi-pi interactions and dipole stacking) define an axis rather than a point. In these cases two interactions may be sufficient for chiral recognition. (Pirkle and Pochapsky (1989), Davankov (1989)). Davankov has also considered the case where diastereomeric analyte-selector complexes formed in the mobile phase interact with the achiral stationary phase. In such a case, two-point analyte-selector interaction may be sufficient, providing the diastereomeric complexes differ in their interactions with the stationary phase.

While an undoubted over-simplification, the three-point attachment rule has proved useful in rationalising many chiral separations. Indeed, considerations of this type were instrumental in development of several of the synthetic chiral stationary phases now widely used. For natural chiral resolving agents, such as proteins and celluloses, application of the three-point rule is more difficult, as the analyte binding site is less clearly defined. In such cases, more empirical development has occurred.

The relationship of enantioselectivity to the thermodynamics of chiral recognition has been investigated (Pirkle and Pochapsky (1989), Boehm *et al* (1988)). Equation 1.1 describes the dependency of chromatographic selectivity, α, on the difference in free energies of formation of the analyte-selector complexes, $\Delta(\Delta G)$

$$\Delta (\Delta G)^{\circ} = -RT \ln \alpha \qquad \text{(eqn. 1.1).}$$

Because of the logarithmic nature of this relationship, small free energy differences result in large α values. Thus, a $\Delta(\Delta G)$ value of 0.25 kJ mol^{-1} gives a chromatographic selectivity of about 1.1. The efficiency of modern chromatographic techniques is such that this degree of selectivity should allow for baseline resolution of the enantiomers, under suitable conditions.

1.2.3 Chiral separations by ligand exchange

Separation of enantiomers may be achieved in chromatographic systems in which the analyte forms a mixed complex with a metal ion and a chiral chelating agent, either added to the mobile phase or immobilised on the stationary phase. This approach was first used to separate amino acid enantiomers on resins loaded with optically active amino acids and transition metal ions. (Davankov *et al* (1973)). The principle behind this method is illustrated in Figure 1.5

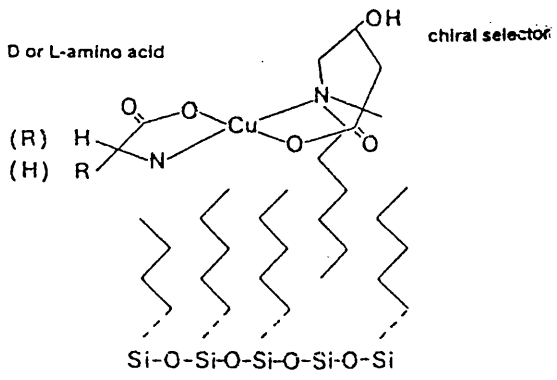


Figure 1.5. Interaction of amino acid enantiomers with Cu^{2+}-L-hydroxyproline bound to silica (from Mack and Hauck (1988))

Three points of interaction may readily be identified. Two are the dative bonds to the copper ion, formed by both analyte enantiomers. The third interaction is stereoselective and involves

steric or hydrophobic interaction between the side-chain moieties of the analyte and selector (Gilon (1981)).

Chiral eluents containing copper ions and amino acids have also been employed (Gil-Av *et al* (1980)). The separation mechanism is similar to that described above.

A number of chiral HPLC ligand exchange phases are commercially available. Ligand-exchange TLC has also been used for separation of enantiomers (Mack and Hauck (1988)) and recently commercialised. The application of chiral LEC has been limited mainly to the resolution of amino acid enantiomers, owing to the relatively specific structural requirements of the chiral recognition mechanism.

1.2.4 Synthetic multiple interaction ("Pirkle"-type) HPLC chiral stationary phases

These stationary phases were devised with the three-point attachment rule firmly in mind (Pirkle and House (1979)). A typical CSP of this type is illustrated in Figure 1.6, which also shows the analyte-selector interactions potentially responsible for the reported resolution of Ibuprofen enantiomers on this phase.

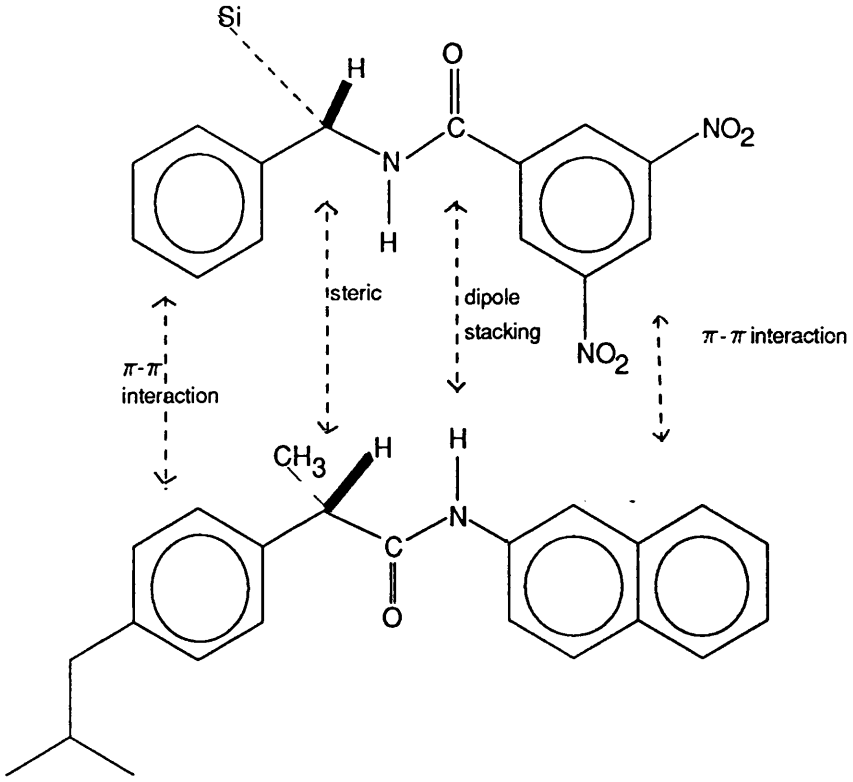


Figure 1.6 Interaction of 3,5-dinitrobenzoylphenylglycine-silica stationary phase with ibuprofen-β-naphthylamide (after Nicoll-Griffith (1987))

CSP's of this type generally have at least one of each of the following types of functional group close to the chiral centre: (i) pi-acidic or pi-basic aromatic groups, capable of charge-transfer interactions (ii) polar hydrogen-bonding or dipole-dipole interaction sites and (iii) bulky non-polar groups to provide steric or hydrophobic interactions.

For successful resolution, the analyte should contain groups which are complementary to the CSP functionalities. As for ibuprofen above, this more often than not requires derivatisation, particularly to introduce the necessary pi-acid or pi-basic site. This requirement is one of the major factors limiting the applicability of these phases, although achiral derivatisation is not as problematic as chiral derivatisation (discussed earlier). The other practical limitation of Pirkle-type CSP's is that they are usually operated in normal-phase mode. Consequently, they are not generally suitable for bioanalytical applications, or for very water-soluble compounds.

A wide variety of multiple-interaction CSP's are now commercially available and, despite the limitations mentioned above, they have been widely used and can be highly selective (Armstrong (1987), Oi and Kitahara (1983)).

1.2.5 Chiral separations using proteins

As has already been discussed, it is well established that the binding of many pharmaceutical agents to proteins - particularly to α_1-acid glycoprotein (AGP) and serum albumin (HSA) - is stereoselective. This has been exploited in the development of HPLC chiral separations using these and other proteins, either immobilised onto silica, or used as mobile phase additives.

Bovine serum albumin (BSA), immobilised onto agarose, was first used to produce a CSP of this type (Stewart and Doherty (1973)). A silica-based BSA phase has since been commercialised as "Resolvosil" (Allenmark *et al* (1983)). Because of the nature of the BSA binding site, the application of this CSP is limited to neutral and anionic molecules. Racemates of pharmaceutical importance, including benzodiazepinones and warfarin (Allenmark (1986)), have been resolved on this phase.

CSP's produced from AGP have complementary application to BSA phases, since AGP selectively binds cationic species (Hermansson (1983)). Thus, a large number of basic pharmaceuticals, including ß-blockers, pheniramines and opioids, have been resolved on AGP phases (Hermansson, J., presented at *2nd Int. Symposium Chiral Sepns.*, Guildford (1989)). Other proteins, including ovumucoid (Miwa *et al* (1987)) and α-chymotrypsin (Wainer, I.W., presented at *2nd Int. Symp. Chiral Sepns.*, Guildford (1989)), have been used to produce CSP's. These have yet to be commercialised, however.

Protein-based CSP's have shown great potential in pharmaceutical applications. They may be operated in reversed-phase mode, and hence may be used for biofluid analysis. The

mechanism of chiral recognition is not clearly defined, however. Method development can therefore only be conducted on something of a "trial-and-error" basis at present. AGP and BSA have also been used as eluent additives (Hermansson (1984)), but economic considerations have deterred most workers from this approach.

1.2.6 Chiral ion-pairing agents

Organic acids and bases are often chromatographed in reversed-phase systems by the addition of a counter-ion to the mobile phase to form "ion-pairs". This approach has been extended to chiral separations, using chiral counter-ions such as (+)-10-camphorsulphonic acid or benzoxycarbonylglycidylproline (ZGP) (Pettersson and Schill (1981)). In order to form tight enough ion-pairs in the mobile phase to give chiral recognition, it is necessary to carry out such separations in the absence of protic solvents, *i.e.* in normal-phase mode. This fact has tended to limit the pharmaceutical applications of this method.

1.2.7 Cellulose, cyclodextrin, crown ether and other CSP's

A wide range of chiral stationary phases based on cellulose are now commercially available. Cellulose is a high molecular-weight polymer of D-(+)-glucose with a helical structure, and can give rise to chiral recognition by means of hydrogen-bonding interactions and inclusion of the solute molecules. Various attempts have been made to prepare cellulose HPLC phases. These have either involved preparation of microcrystalline cellulose derivatives or coating of cellulose derivatives onto silica (Okamoto *et al* (1984)). The latter phases have found wider application because they are better able to withstand high-pressure operation. About a dozen silica-cellulose ("Chiracel") phases are now commercially available, with distinct selectivity according to their functionality. They are operated in normal phase mode, and are not very robust, with certain solvents tending to "strip off" the cellulose coating from the silica (Johns (1989)). Their high cost has also limited their use.

Cyclodextrins are also glucose oligomers, and give rise to chiral recognition via inclusion complexation (Hinze (1981)). Particularly with the introduction of derivatised cyclodextrin CSP's for use in normal-phase mode (Astec (1991)), cyclodextrin-silica phases may supercede cellulosic phases in many applications, as they are chemically bonded phases (and hence more robust) and are considerably cheaper than Chiracel columns. Their use, both in CSP's and as eluent additives, will be discussed fully in subsequent chapters.

A stationary phase incorporating a chiral crown ether (Crownpak) has recently been commercialised. The mechanism of chiral recognition in this case is also thought to involve inclusion complexation.

A variety of synthetic chiral polymers have been immobilised onto silica and used as CSP's (Pirkle and Pochapsky (1989)). Typical of these is poly(triphenyl methyl methacrylate), a helical polymer which forms the basis of the recently marketed Chiralpak OT phase (Okamoto *et al* (1984)). The mechanism of chiral recognition on such phases may be similar to that on cellulose, *i.e.* a mixture of polar and steric interactions.

1.2.8 Preparative HPLC chiral separations

The development of chiral chromatography has so far been mainly limited to analytical scale separations. However, owing to the increasing demand for production of optically pure drugs, preparative chromatographic resolution of enantiomers, based on the methods discussed above, is likely to assume greater importance (Pirkle and Hamper (1987), Meyer (1987)).

Pirkle and Hamper (1987) have discussed the requirements for such separations. For high throughput, large samples must be introduced to the column. Under such overloaded conditions, both selectivity and efficiency are lower than when column loading is small. It is therefore critical to obtain high selectivity under analytical conditions for efficient preparative separations. It is also important that the loss in efficiency of the CSP with increasing sample

size is minimised, *i.e.* that the phase has high loadability. A typical achiral silica or reversed phase packing has a loadability of the order of 1mg sample on column per gram of packing material (Done (1976)). Many of the currently available CSP's have considerably lower capacity than this, and this limits their preparative applicability.

The separation of gram quantities of material in reasonable time usually involves the use of large sized stationary phase particles and high flow rates, using large columns (500x20mm and greater). Under such conditions, column efficiencies are lower than under analytical conditions, but the large particles may have greater loadability than the 5µm particles used in analytical columns. Milligram quantities of enantiomers, where throughput is not as critical, are more usually separated on semi-preparative columns of 10mm in diameter, packed with 5-10µm particle stationary phases.

The protein-based CSP's, where high molecular weight limits the concentration of binding sites on the stationary phase, show perhaps most clearly the limitations imposed by low loadabilities. Erlandsson *et al* (1986) investigated the preparative resolution of tryptophan, oxazepam and benzoin enantiomers on a BSA-silica column. Very high selectivities (3.0, 2.1 and 1.7, respectively) were obtained at low column loading, giving more than baseline resolution in all cases. However, resolution decreased very rapidly on increasing the sample size up to 1mg on a 500x22mm column, and throughputs of only 1mg/hour at high optical purity could be achieved in the best case.

The cyclodextrin bonded phases are also reported to have low capacity (approximately 25% of that of a conventional reversed-phase packing), due to low coverage of the cyclodextrin on the silica (Vigh *et al* (1989a)). However, impressive separations have recently been achieved on these columns in displacement mode (Vigh *et al* (1989), (1989a), (1990)).

The CSP's that have found greatest preparative application are the Pirkle-type phases,

owing to their high selectivity for many pairs of enantiomers, and to their high capacity. The loadability of DNB-phenylglycine-silica is of the same order as a conventional silica column, with mass overload occurring at above 200µg sample on column per gram of packing material (Meyer (1987)).

The preparative resolution of chiral benzodiazepinones was demonstrated by Pirkle and Tsipouras (1984). Selectivities of over 4.0 on (S)-3,5-DNB-leucine-silica were achieved for some of the compounds studied. In such cases, 100mg racemate could be resolved into enantiomeric fractions at 99% optical purity on a 250x10mm column packed with 5µm material at a single pass.

Pirkle and Hamper (1987) studied in detail the variation in resolution of enantiomers of 2,2,2-trifluoro-1-(9-anthrylethanol) with sample loading on an analytical (250x4.6mm) column packed with 3,5-DNB-phenylglycine covalently bonded to 5µm aminopropyl-silica, using a mobile phase consisting of 2% propan-2-ol in hexane at 1 - 2ml/min. While only moderate selectivity (α = 1.44) was seen at low column loading, the capacity and efficiency of the phase was such that useable resolution was obtained, even at very high column loading, as shown in Figure 1.7.

An R_s value of 1, which theoretically corresponds to 98% purity of each fraction (Snyder (1972)), was obtained at 5.4mg column loading. The separation was scaled-up by a factor of 4.7 by using a 250x10mm column. Under these conditions, 25mg racemate was resolved and the predicted 98% enantiomeric excess obtained in each enantiomer fraction. Higher throughputs were obtained by increasing the loading still further, and using a second pass through the system to obtain high optical purities.

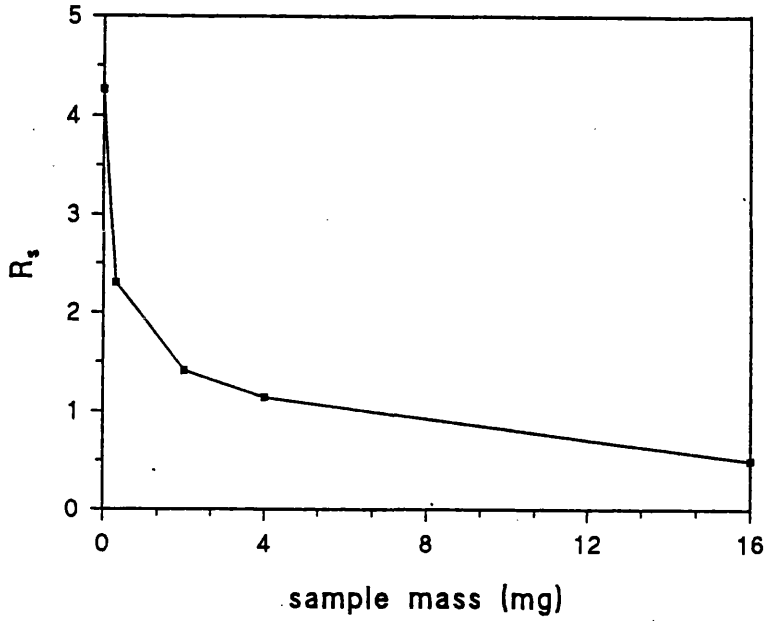


Figure 1.7. Variation in resolution of enantiomers of 2,2,2-trifluoro-1-(9-anthryl)ethanol on Pirkle column. Conditions as text. (After Pirkle and Hamper (1987))

Pirkle has reported that CSP's such as that above consistently exhibit capacities of 5mg solute per gram of packing material. Pirkle columns are now available commercially in large sizes packed with large particles for preparative work. The availability of both antipodes of a column type enables the manipulation of elution order. Perry and Rateike (1990) have shown that elution of the trace component first is advantageous. The main problem with these phases is the need for achiral derivatisation before separation, which introduces complications in recovery of the resolved enantiomers.

Several preparative separations on Chiracel cellulosic phases have been reported. The enantiomers of prostaglandin precursors were resolved at a throughput of 400mg/hour of the required enantiomer on a 500 x 20mm Chiracel OC (cellulose triphenylcarbamate) column (Miller and Bush (1989), Miller and Weyker (1990)). A similar Chiracel OC column was used to resolve enantiomers of ß-blocking drugs at 100mg scale. (Okamoto (1986)). Masurel and

Wainer (1989) reported rapid mg-scale chiral resolution of ifosfamide on Chiracel OD. It is clear that the loadability of these phases is high enough for preparative applications, but at present the commercial cost of large columns of this type is very high.

The use of mobile phase additives to achieve preparative chiral separations is not widely reported. This is largely due to the difficulty of recovering the resolved enantiomers free of the resolving agent, and because of the high cost of the large quantities of the additive required (Smith, R.M., presented at *2nd. Int. Symp. Chiral Sepns.,* Guildford (1989)). Sellergren *et al* (1988) reported the use of ultrafiltration for the removal of BSA from D- and L-kynurenine following semi-preparative separation by counter-current extraction. This technique might be used for other macromolecular chiral additives, and allowed the recovery of the additive for re-use.

Preparative separation of amino acid enantiomers by ligand exchange using mobile phase additives has also been reported (Jeanneret-Gris *et al* (1989), Keller and Niwa (1989)). Removal of the additives (copper ions and amino acids) from the product was achieved in both cases by ion-exchange.

1.3. Cyclodextrins

1.3.1 Preparation, structure and physical properties

Cyclodextrins (CDs) are a series of glucose oligomers produced by the action of *Bacillus macerans* amylase (and certain other microbial amylases) on starch. They were first isolated from degraded starch by Villiers (1891), and were characterised as cyclic oligosaccharides by Schardinger (1904). Cyclodextrins consist of six to twelve α-D-glucopyranose units in a ring joined by (1 - 4) linkages. The members of the series formed in highest yield from starch are the alpha-, beta-, and gamma- oligomers, containing six, seven, and eight glucose residues, respectively. The structure of beta-cyclodextrin is shown in Figure 1.8.

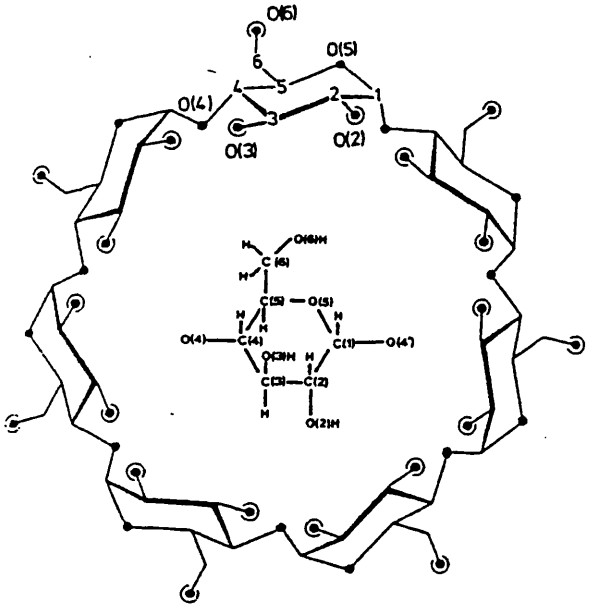


Figure 1.8. Chemical structure of ß-cyclodextrin, with numbering of atoms of glucose unit also shown. • = oxygen atoms. ⊜ = hydroxyl groups. (From Saenger (1980))

X-ray crystallographic studies (Takeo and Kuge (1970)) on solid cyclodextrins, and NMR and circular dichroism studies of their aqueous solutions (Wood *et al* (1977)) have shown that the

glucose units are in the C1 chair conformation, and that hydrogen bonds are formed between 2- and 3- hydroxyl groups of adjacent glucose units. These hydrogen bonds are thought to confer conformational rigidity on the molecule, and are maintained even in aqueous solution. As shown in Fig. 1.9, cyclodextrins are shaped like hollow cones , with the primary (6-) hydroxyls at the narrower face and the secondary (2- and 3-) hydroxyls at the wider end of the cavity. The cavity contains the ether-like 4- oxygen atoms and the H-5 and H-3 protons of the glucose units, and is therefore relatively hydrophobic compared to the external faces of the molecule where the hydroxyl groups are situated (Griffiths and Bender (1973), Street (1987), Hinze (1981)).

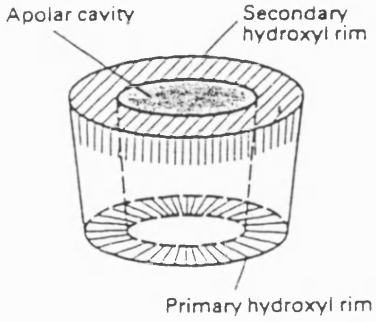


Figure 1.9. The molecular shape of ß-cyclodextrin (From Pagington (1987))

The physical properties of the common cyclodextrins are summarised in Table 1.1, overleaf.

The anhydrous forms are somewhat deliquescent, forming one of several hydrates, depending on temperature. Wiedenhoff and Lammers (1968) found ß-cyclodextrin hydrate to contain 12 mole equivalents of water at 15-30^{o}C. The aqueous solubilities of the cyclodextrins were investigated by Jozwiakowski and Connors (1985). They found that dissolution in water was enthalpically unfavourable but entropically favourable. Hence, solubility was significantly enhanced on raising temperature. It is not clear why ß-cyclodextrin is so much

less soluble in water than the other oligomers. ß-cyclodextrin is even less soluble in most organic solvents than in water (Saenger (1980)), but is freely soluble in dimethylsulphoxide and dimethylformamide.

Table 1.1. Physical properties of cyclodextrins (from Saenger (1980), Hinze (1981))

CD	MW	Cavity dimensions, Angstroms			aq.soly.
	(anhydrous)	ext. diameter	int. diameter	depth	%w/v (25°C)
alpha-	973	14.6	4.7 - 5.2	4.5	14.5
beta-	1135	15.4	6.0 - 6.4	7	1.85
gamma-	1297	17.5	7.5 - 8.3	7	23.2

Cyclodextrins are fairly stable compounds, decomposing on heating (before melting) above 200°C (Szejtli (1982)). Like all saccharides, they are susceptible to hydrolysis by strong acids, but are stable in alkaline media (French and McIntire (1950)). The hydroxyl groups have relatively low pKa values, between 12.1 and 12.6 (VanEtten *et al* (1967)), and may be deprotonated at high pH, leading to increased aqueous solubility (Pharr *et al* (1989)).

1.3.2. Cyclodextrin inclusion complexes

The most significant property of cyclodextrins is their ability to form inclusion complexes in aqueous solution. This was first reported by Freudenburg and Cramer (1948). A wide range of guest molecules have been shown to form such complexes, from noble gases (Saenger (1980)) and inorganic anions (Rohrbach *et al* (1987)) through simple aromatic molecules (Lewis and Hansen (1973)) to large species such as steroids (Gazdag *et al* (1986)).

Complex formation is frequently evidenced by changes in the physical and chemical properties of both guest and host, as the portion of the guest included in the cavity is moving

from aqueous solution into a more hydrophobic environment inside the cavity. This may cause changes in the NMR spectra of both guest and host (Demarco and Thakkar (1970)). Fluorescence enhancement on complexation with cyclodextrins has been shown to occur for solutes such as 1-anilino-8-naphthalenesulphonate (Cramer *et al* (1967)) and dansyl amino acids (Kinoshita *et al* (1973)). Changes in guest UV absorption (Otagiri *et al* (1976)) and circular dichroism have also been reported (Uekama *et al* (1977), Kajtar *et al* (1981)). Inclusion also results in changes in the pKa values of ionisable guests, since ionised guests are generally less strongly included in the hydrophobic cavity (Connors and Lipari (1976)). Complexation often results in solubilisation of the guest in water, since the hydrophobic portion of the included species is less exposed to the solvent. This effect has been demonstrated for a number of poorly soluble pharmaceuticals, notably barbiturates (Thakkar *et al* (1972)), non-steroidal anti-inflammatory agents (Hamada *et al* (1975)) and prostaglandins (Hatachi and Inaba (1974))..

The variation in these physical properties with cyclodextrin:substrate ratio have been widely used to estimate the stoichiometry and stability of cyclodextrin complexes (Connors and Lipari (1976), Gelb *et al* (1981), Miyaji *et al* (1976), Cramer *et al* (1967), Bergeron *et al* (1977a), Ikeda *et al* (1975)), using methods such as those of Job (1928) and Benesi and Hildebrand (1949). In most cases, complex stoichiometries are 1:1, but 2:1, 1:2 and even higher order stoichiometries have been reported (Szejtli (1982)). Complex formation (equilibrium) constants of up to 10^4 have been reported (Otagiri *et al* (1975)).

Complexation may also cause changes in the chemical properties of a guest molecule. For example, the stability of phenothiazines towards oxidation is greatly increased by inclusion in ß-cyclodextrin (Otagiri *et al* (1975)). Cyclodextrins have also been shown to catalyse certain reactions, such as prostaglandin isomerisation (Uekama *et al* (1978)) and dechlorination of chlorpromazine (Uekama *et al* (1978a)). .

1.3.3 Thermodynamics of inclusion complex formation

The thermodynamics of a number of cyclodextrin complexation reactions have been investigated. Free energy, enthalpy, and entropy of complex formation may be determined from the varation of complex stability with temperature (Cramer *et al* (1967)), or by microcalorimetry (Lewis and Hansen (1973). Typical data from these studies are given in Table 1.2 (overleaf). In all cases, complexation is enthalpically favourable (*i.e.* ΔH is negative). The entropic contribution to the free energy of complexation at 273K is generally small. ΔS is more often negative than positive.

The driving forces for formation of cyclodextrin inclusion complexes have been discussed widely. Tabushi *et al* (1978) have devised a model of the complexation process, which is illustrated in Figure 1.10.

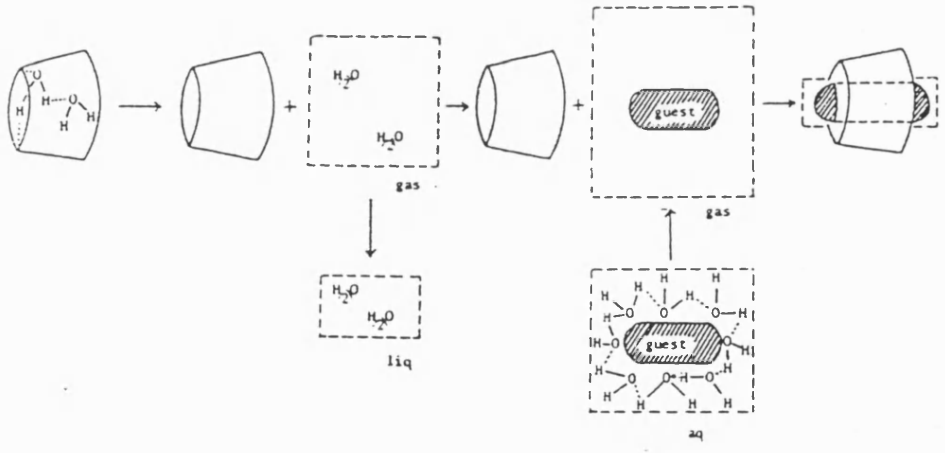


Figure 1.10. Schematic representation of the thermodynamic process of inclusion of an organic guest molecule by alpha-cyclodextrin in aqueous solution. (from Tabushi *et al* (1978))

Tabushi argued that the overall stabilisation due to complexation can be estimated by summation of the free energy changes due to (i) release of "gaseous" water molecules from

Table 1.2. Thermodynamic parameters (in KJ mol^{-1}) for formation of cyclodextrin complexes at 298k

Guest	Cyclodextrin	K_f, M^{-1}	ΔG_f	ΔH_f	$T\Delta S_f$	method	ref
benzene	alpha		-21.7	-16.7	2.1	calc	Tabushi (1978)
p-iodoaniline	alpha		-37.6	-30.8	6.9	calc	Tabushi (1978)
benzoic acid	alpha			-42.5	-26.5	pot	Gelb (1981)
benzoate anion	alpha			-16.3	-10.5	pot	Gelb (1981)
p-nitrophenol	alpha			-30.5	-17.2	spec	Gelb (1981)
		220	-13.4	-25.8	-12.5	mc	Bertrand (1989)
p-nitrophenol	beta	350	-14.5	-12.0	2.4	mc	Bertrand (1989)
p-nitrophenolate	alpha			-46.9	-27.4	spec	Gelb (1981)
p-cyanophanol	alpha	140		-25.5	-13.0	pot	Gelb (1981)
m-cyanophenol	alpha	95		-25.5	-14.3	pot	Gelb (1981)
phenobarbital	beta	1690		-43.1	-25.1	pot	Miyaji (1976)
phenobarbital sodium	beta	192		-27.6	-15.1	pot	Miyaji (1976)
D-norleucine	alpha		- 9.5	- 9.3	0.2	mc	Barone (1989)
L-norleucine	alpha		- 9.5	- 8.9	0.6	mc	Barone (1989)
D-phenylalanine	alpha	18		-16.3		mc	Cooper (1978)
L-phenylalanine	alpha	15.5		-15.5		mc	Cooper (1978)
(+)-2-phenylethylamine	alpha			-15.1		mc	Cooper (1978)
(-)-2-phenylethylamine	alpha			-15.5		mc	Cooper (1978)
D-mandelate anion	alpha	7.8		-13.0		mc	Cooper (1978)
L-mandelate anion	alpha	7.8		-14.2		mc	Cooper (1978)

mc = microcalorimetry; pot = potentiometry; sol = solubility; spec = spectrophotometry; calc = energy calculation

the cavity, (ii) return of water molecules to the bulk liquid, (iii) transfer of guest from solvated to "gaseous" state, with collapse of the structured water cavity it occupied, and (iv) binding of the guest in the cyclodextrin cavity. He calculated the enthalpy and entropy changes associated with each stage for the complexation of benzene, p-iodoaniline, and methyl orange by ß-cyclodextrin. In each case, the major driving forces for complexation were found to be host-guest Van der Waals forces (from stage iv) and stabilisation of the water molecules expelled from the cavity (at stage ii). These factors were discussed by Saenger *et al* (1976). The water molecules inside the cavity in the solvated host were termed "high-energy" water, since they cannot form their full complement of hydrogen-bonds. On expulsion from the cavity and return to the bulk solvent, these water molecules are stabilised by being able to form additional H-bonds. The loss in host-water Van der Waals stabilisation may be more than compensated for by Van der Waals attraction between the host and the hydrophobic guest.

Water stabilisation is independent of the nature of the guest. Thus, differences in the stabilities of complexes formed by a given cyclodextrin reflect mainly the size of the host-guest Van der Waals forces, which depend on the "fit" of the guest in the cavity. Thus, benzene derivatives are well known to complex strongly with alpha-cyclodextrin, but larger aromatic molecules such as naphthalene complex more strongly with the larger beta-cyclodextrin. Steroidal multi-ring molecules only complex strongly with gamma-cyclodextrin (Szejtli (1982), Gazdag *et al* (1986)).

Mularz (1988) has demonstrated most elegantly the effect of guest size on complex stability. He investigated the complex stabilities of several series of halogenated benzene derivatives with ß-cyclodextrin, and found linear relationships between the molar volume of the halogen substituents and the complex stabilities. The importance of steric factors is also illustrated by the data for meta- and para-cyanophenol (Table 1.2), where position of substituents on the benzene ring is seen to have a marked influence on complex stability. Table 1.2 also shows

the effect of acid ionisation on complex stability. In general, carboxylic acids are more strongly complexed as their protonated forms, but the reverse is often true of phenols.

The strength of Van der Waals interactions in the cavity also depend on the hydrophobicity of the guest. This would account for the close correlation observed between complex stabilities and octanol-water partition coefficients within a structurally related series (Otagiri *et al* (1975)), and for the observation that inclusion of ionised and strongly polar groups is generally disfavoured (Szejtli (1982))

Szejtli (1982) has pointed out that the relative effects of water stabilisation and Van der Waals interactions in providing a driving force for complexation may vary with cyclodextrin ring size. Thus, in gamma-cyclodextrin the water molecules in the larger cavity are more like the bulk solvent in terms of their hydrogen-bonding capability than in the small alpha-cyclodextrin cavity. Expulsion of high-energy water might therefore be expected to contribute less to overall stabilisation of gamma-cyclodextrin complexes.

Saenger *et al* (1976) also suggested that relief of ring-strain is a driving force for formation of alpha-cyclodextrin complexes. Tabushi's calculations, however, suggest that conformational changes if anything detract from complex stability. Bergeron *et al* (1977a) found no NMR evidence for significant changes in host geometry on complexation. Guest-host hydrogen-bonding has been suggested to account for the differences in stabilities of complexes of enantiomeric guests (Gelb *et al* (1981), Otagiri *et al* (1976)). However, Tabushi's calculations do not suggest that such interactions play an important role in overall complex stabilisation. The evidence for such interactions in solution is sparse.

The small, and generally unfavourable, changes in entropy on complex formation were thought by Tabushi to arise from a near-cancelling out of reduced guest entropy due to loss of rotational degrees of freedom on inclusion against the increased entropy of the solvent on

being expelled from the cavity.

1.3.4 Stereospecificity of cyclodextrin inclusion

Being oligomers of D-glucose, cyclodextrins are homochiral. ß-cyclodextrin, for example, contains 35 chiral centres. Complexes formed with the two enantiomers of a chiral guest are therefore diastereomeric. Such diastereomeric complexes may differ in their physical and chemical properties, such as solubility, solid state structure, UV, CD, NMR and other spectra, chemical reactivity, and chromatographic retention.

Stereoselective inclusion by cyclodextrins was first noted by Cramer and Dietsche (1959), who resolved the racemates of a number of aromatic acids and their esters by fractional crystallisation of their beta-cyclodextrin complexes. Cramer and Dietsche (1959a) also used cyclodextrins to catalyse enantioselective hydrolysis of chiral esters.

Cooper and MacNicol (1978) measured the enthalpy changes on complexation of a number of enantiomeric solutes with alpha-cyclodextrin. Some of their results are listed in Table 1.2. In some cases (*e.g.* phenylalanine), significant differences in the stabilities of the diastereomeric complexes were seen, but the precision of the experiments was not sufficiently great to allow assessment of whether chiral discrimination arose due to enthalpic or entropic effects. They suggested that chiral discrimination was arising due to differences in the binding of the exposed guest substituent groups to the cyclodextrin hydroxyls around the rim of the cavity, and that cavity interactions (which provide the main driving force for inclusion) were not enantioselective. This suggestion has also been made by R.D. Armstrong (1987) to account for the discrimination between propanolol enantiomers induced by ß-cyclodextrin. Molecular modelling studies showed that the polar groups of one enantiomer were so orientated that they could form an extra hydrogen bond with the cyclodextrin primary hydroxyls. While in this case, the relative degree of interaction of the two enantiomers with

the substrate were correctly predicted qualitatively, quantitative studies in this area are lacking at present.

The differences in solid state structures of diastereomeric cyclodextrin complexes have been investigated by X-ray diffraction for a number of chiral guests, including flurbiprofen (Uekama *et al* (1983)), fenoprofen (Hamilton and Chen (1988)) and mandelic acid (Harata *et al* (1984)). However, it seems unlikely that these results have much relevance to the study of chiral discrimination in solution. Complex stoichiometries are rarely 1:1 in the solid state. Cyclodextrin molecules tend to associate to form "channels" in the crystal lattice, and "guest" molecules are often found in the channels as well as in the cyclodextrin cavities (Saenger (1984)). Solvation interactions in solution are likely to be very different to lattice interactions in the solid state.

1.3.5 Modified cyclodextrins

A wide range of derivatives of the native cyclodextrins have been produced by chemical synthesis (Croft and Bartsch (1983)). These have served to broaden the application of cyclodextrins.

Of particular note are the methylated cyclodextrins - 2,6-di-O-methyl- and 2,3,6-tri-O-methyl-(permethyl). In the former, the primary and half of the secondary hydroxyls are methylated. In the latter, all the hydroxyl groups are methylated. The aqueous solubility of these derivatives is significantly enhanced relative to the parent cyclodextrin (Casu *et al* (1979)), which is of particular significance for the otherwise poorly soluble ß-oligomer. Methylation of the secondary hydroxyl groups is thought to enhance solubility by disrupting intramolecular hydrogen-bonding, allowing more water-cyclodextrin solubilising interactions.

The inclusion properties of methylated cyclodextrins have been shown to be different from those of the parent cyclodextrins. A number of authors (Szeman *et al* (1987), Uekama *et al*

(1985), Casu *et al* (1979)) have shown that dimethylated cyclodextrins form more stable complexes than the parents with a number of pharmaceuticals. Trimethylated cyclodextrins appear to form weaker complexes in most cases. (Nakai *et al* (1983), Otagiri *et al* (1984), Imai *et al* (1988), Uekama *et al* (1985)). Chiral recognition is often particularly strong in trimethylated cyclodextrin complexes (Imai (1988)). This has been attributed to the more distorted shape of the trimethyl-cyclodextrin cavity allowing more stereospecific interactions inside the cavity (Harata (1990), Uekama *et al* (1985)).

Hydroxyalkylated cyclodextrins, particularly hydroxypropyl-, have come to the fore in recent years. They also are highly soluble in water, and have been widely used as solubilising agents for pharmaceuticals (Muller and Brauns (1985)). They do not exhibit the toxicity of methylated cyclodextrins, and have similar complexing ability as the parent cyclodextrins, particularly at low degrees of substitution (Pitha and Pitha (1986)).

3.1.6 Applications of cyclodextrins

Cyclodextrins have found wide application in chemistry, and in the pharmaceutical, food and other industries. The ß-oligomer and its derivatives have been most widely used, partly due to favourable complexing properties and also to economic considerations. However, the prices of all cyclodextrins, and particularly the previously prohibitive gamma-cyclodextrin, have fallen markedly in the last few years, opening up greater possibilities for their application.

(a) Pharmaceutical applications

The potential use of cyclodextrins to solubilise and/or stabilise pharamaceutical agents has already been mentioned. The bioavailability of a number of drugs has been shown to be markedly increased as a result of the additional solubility of their complexes, and it seems likely that cyclodextrin-containing formulations may be widely used in the future. One

commercially available formulation of this type is Prostarmon, the ß-cyclodextrin-complex of Prostaglandin E_1, which has enhanced stability properties relative to the uncomplexed drug (Jones *et al* (1984a)).

(b) Applications in the food industry

ß-cyclodextrin is approved for use as a food additive in several countries. It potentially may be used as a stabilising agent, such as in Vitamin D_3 preparations, and may help to retain volatile flavour and aroma components. Cyclodextrins may also be used to extract desirable or undesirable components from foodstuffs, such as free fatty acids from vegetable oils (Pagington (1987)).

(c) Enzyme models

Cyclodextrins are good, if simple, models for enzyme active sites, as they rely on steric recognition for complex formation. They have therefore been widely used in studies of enzyme kinetics and competitive binding (Bergeron (1977)).

(d) Analytical applications

The applications of cyclodextrins in analytical chemistry have recently been reviewed (Armstrong (1988)). The use of cyclodextrins to enhance fluorescence has already been mentioned, and has been utilised to increase detectability of dansyl amino acids (Kinoshita *et al* (1973)). Their effect on NMR spectra of included guests, particularly in inducing chiral discrimination, has led to their use as NMR shift reagents (to be discussed in Chapter 5).

Cyclodextrins have been widely used in chromatographic and related techniques (notably gel chromatography, HPLC, gas chromatography, super-critical fluid chromatography, isotachophoresis, and electrophoresis). Incorporated in stationary or mobile phase, they impart distinct selectivity to the chromatographic process through inclusion interactions. Of particular

interest has been the possibility of achieving chromatographic chiral separations using cyclodextrins. This will be discussed in more detail in Chapters 3 and 4.

Chapter 2

Experimental

2.1 Materials

N.B. : All materials used as received, unless otherwise stated. All chiral compounds used were racemic, unless otherwise stated.

2.1.1 Solvents, HPLC buffer components and ion-pairing agents

HPLC grade methanol (MeOH), acetonitrile (ACN), tetrahydrofuran (THF), acetone, chloroform, triethylamine (TEA), trifluoroacetic acid (TFA), hexanesulphonic acid sodium salt (SHS) and tetra-^{n}butylammonium bromide (TBA) were obtained from FSA, Loughborough.

Analytical Reagent grade dimethylsulphoxide (DMSO), disodium hydrogen orthophosphate dihydrate (Na_2HPO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), sodium hydroxide (NaOH), sodium acetate (NaAc), and orthophosphoric acid SG 1.69 (H_3PO_4) were obtained from FSA, Loughborough.

GPR grade anhydrous sodium carbonate (Na_2CO_3) was obtained from BDH, Poole. Sodium Decyl Sulphate (SDS) was obtained from Cambrian Chemicals (Croydon, Surrey). SLR grade glacial acetic acid (HAc), 0.880 ammonia solution (NH_3) and anhydrous magnesium sulphate ($MgSO_4$) were obtained from FSA, Loughborough.

Ether (SLR grade) was obtained from FSA, Loughborough, and was distilled over sodium/ benzophenone before use. Water for HPLC was single distilled and passed through 0.45µm cellulose nitrate membrane filters before use.

2.1.2 Cyclodextrins

ß-cyclodextrin hydrate (containing 10-13% water, w/w) was obtained from Aldrich (Gillingham, Dorset), Sigma (Poole), Lancaster Synthesis (Morecambe, Lancs.), Janssen Chimica (Beerse, Belgium) or Wacker Chemie GMBH (Munich, Germany). Alpha and gamma

cyclodextrins were obtained from Aldrich.

Derivatised cyclodextrins were characterised in terms of degree of substitution (DS) or molar substitution (MS), equivalent to the average number of substituent groups per glucose unit.

Methyl-ß-cyclodextrin (Me-ß-CD) (DS = 1.8), hydroxyethyl-ß-cyclodextrin (HoEt-ß-CD) (MS = 0.6 or 0.9), and hydroxypropyl-ß-cyclodextrin (HoPr-ß-CD) (MS = 0.9) were obtained from Aldrich, from Technicol (Stockport), or from Wacker-Chemie GMBH (Munich, Germany).

A sample of a hydroxypropyl-ß-cyclodextrin was also obtained from Dr. Bernd Muller, Christian Albrechts University, Kiel, Germany. This was characterised by NMR, and found to have an MS value of about 0.5.

Cyclodextrins for thermodynamic studies were dried for 24 hours at 125°C before use. Otherwise, cyclodextrin hydrates were used as received.

2.1.3 Materials for NMR studies

Deuterium oxide (D_2O), 99.9atom% D, was obtained from Aldrich, from Goss (Ingatestone, Essex), or from MSD Isotopes (Montreal, Canada). Methyl sulphoxide-d_6 (DMSO-d_6) 99.9atom% D was obtained from MSD. Acetone-d_6 (99atom% D, Gold Label), methanol-d_4 (CD_3OD) (99.8atom% D), acetonitrile-d_3 (97% Gold Label), and ethanol-d_1 (EtOD) (99.5+atom% D) were obtained from Aldrich.

Sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) was obtained from BOC (Croydon) and was used as a reference (δ = 0.00ppm) in D_2O. Maleic acid (SLR) was obtained from FSA, and was used as an internal standard in quantitative experiments.

2.1.4 Preparation of ±-trimeprazine trifluoroacetate

±-Trimeprazine hemi-(+)-tartrate (0.5g) was dissolved in distilled water (20ml). Trifluoroacetic acid was added dropwise until trimeprazine trifluoroacetate was produced as a gelatinous white precipitate. This was extracted into chloroform (2 x 10ml) and the organic phase was dried over $MgSO_4$, before evaporation. After drying over P_2O_5 at reduced pressure, a viscous green oil resulted which crystallised over several months. The crude trifluoroacetate was used for NMR comparison with chromatographically resolved material.

2.1.5 Solutes

The following solutes were obtained commercially:

d- and l- mandelic acid (Aldrich, 99% Gold Label); tropic acid (Aldrich, 97%); 3-phenyllactic acid (Nutritional Biochemicals, Cleveland, Ohio, USA); (S)- and (R)-2-methoxyphenylacetic acid (Lancaster Synthesis); (S)- and (R)-O-acetylmandelic acid (Sigma); 2-chloromandelic acid (Janssen Chimica); 4-chloromandelic acid (Lancaster Synthesis); 2- and 3-methoxymandelic acids (Sigma); 4-methoxymandelic acid (Lancaster); 3- and 4-hydroxymandelic acids (Sigma); DL-mandelic acid methyl ester (Sigma); R-(-)- and S-(+)- mandelic acid methyl esters (Aldrich, 99+% Gold Label); mandelic acid ethyl, benzyl and isoamyl esters (Sigma); α-cyclohexylphenylacetic acid (Aldrich, 96%); 2-phenylpropionic acid (Aldrich, 98%); d-(+)-tartaric acid (Aldrich, 99+% Gold Label); hydroxyzine dihydrochloride (Sigma); S-(+)-benzoin (Aldrich, 99%); benzoin (BDH); (-)- and (+)- pseudoephedrine hydrochlorides, (+)- and DL-ephedrine hydrochlorides (Sigma); (-)-ephedrine hydrochloride (BDH); norephedrine hydrochloride (Sigma); L- and DL-phenylalanine (Sigma); DL-3,4-dihydroxyphenylalanine (DOPA) (Aldrich, 97%); D-, L- and DL-trytophan (Sigma); meclizine dihydrochloride (Sigma); trimeprazine hemi-(+)-tartrate (Sigma); and meta-nitroaniline (Aldrich).

Samples of the following compounds were obtained from pharmaceutical research

laboratories or other non-commercial sources:

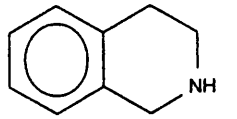
Phenothiazines (trimeprazine hemi-(+)-tartrate, l-methotrimeprazine hydrochloride, l-methotrimeprazine tartrate, d-methotrimeprazine maleate, promethazine hydrochloride and dimethothiazine mesylate (fonazine)) were obtained from Rhône-Poulenc (Dagenham, Essex). Isothipendyl hydrochloride was donated by Astra Pharma AG (Frankfurt, Germany). Tetrahydroisoquinolines (TQ1 - TQ7) were supplied by Dr. G.H. Dewar, University of Bath. ICI Pharmaceuticals (Macclesfield, Cheshire) supplied samples of thromboxane antagonists (TA1 -TA17), tetramisole hydrochloride, ICI1 and ICI2.

SmithKline Beecham (Welwyn, Herts) supplied chlorpheniramine maleate, mebrophenydramine hydrochloride, and fenoldopam. Ciba-Geigy (Horsham, Sussex) supplied methyl phenidate hydrochloride, chlorthalidone, and oxyphenonium bromide (antrenyl). A.H. Robins (Horsham, Sussex) supplied pheniramine maleate and brompheniramine maleate. Wyeth (High Wycombe, Bucks) supplied carbinoxamine maleate. Zyma (Alderley Edge, Cheshire) supplied dimethindene maleate. Pfizer (Groton, Conn., USA) supplied buclizine hydrochloride (vibazine). (+)- and (-)-neobenodine hydrochlorides were obtained from Dept. Pharmacochemistry, Vrije Universiteit, Amsterdam. Disopyramide phosphate was supplied by Hässle AB, Sweden. Benzhexol (trihexyphenidyl) was obtained from Cyanamid (Gosport, Hants.). AM5(6) was obtained from Prof. M. Stevens, Dept. of Pharmacy, Aston University. Riker Labs. (Loughborough) supplied nefopam hydrochloride. Schwarz Pharma AG (Monheim, Germany) supplied bupranolol hydrochloride and bunolol hydrochloride. Tropicamide was obtained from Smith and Nephew (Romford, Essex). Metoprolol tartrate, orciprenaline sulphate and prilocaine hydrochloride were supplied by Astra (Kings Langley, Herts). Salbutamol sulphate was supplied by Glaxo (Ware, Herts). Dexamisole and levamisole hydrochlorides were obtained from Janssen Chimica (Beerse, Belgium). Verapamil hydrochloride was obtained from Abbott Laboratories (Maidenhead). Telemzepine dihydrochloride was obtained from BYK Gulden (Konstanz, Germany).

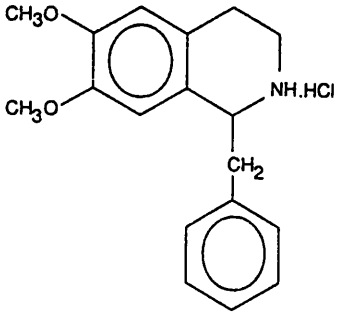
Roche (Welwyn, Herts) supplied benserazide hydrochloride and phenindamine tartrate (thephorin). The latter is a non-racemic mixture consisting of 60-65% d-phenindamine-d-tartrate and 35-40% l-phenindamine-d-tartrate.

2.1.6 Structures of compounds investigated

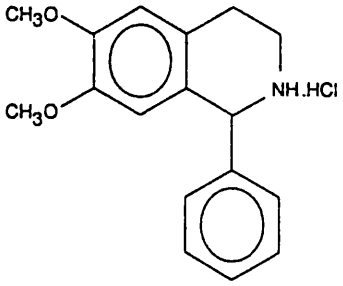
(a) Tetrahydroisoquinolines



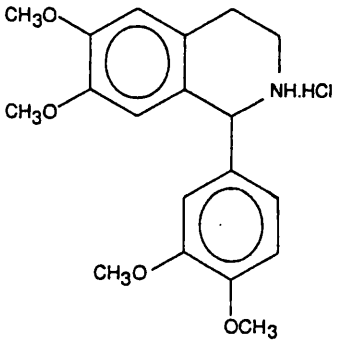
1,2,3,4-tetrahydroisoquinoline



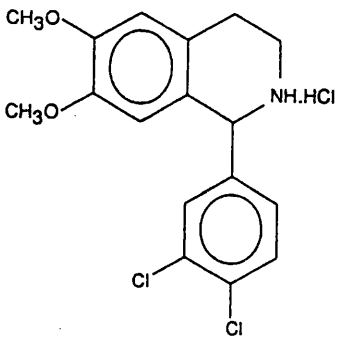
TQ1



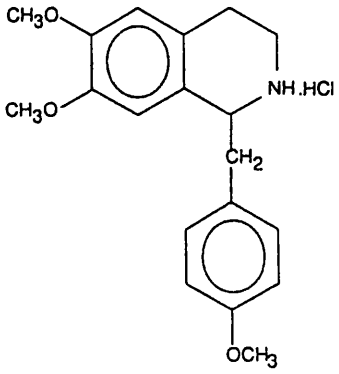
TQ2



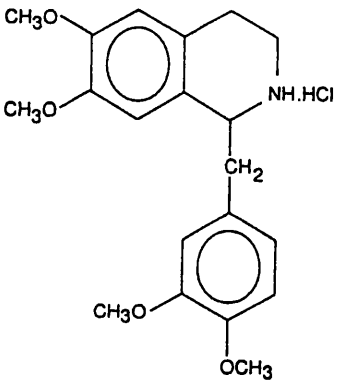
TQ3



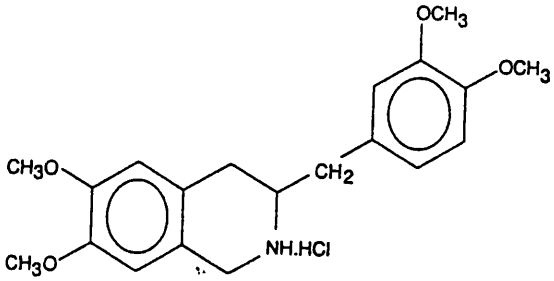
TQ4



TQ5

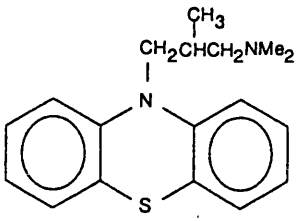


TQ6

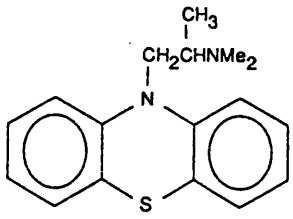


TQ7

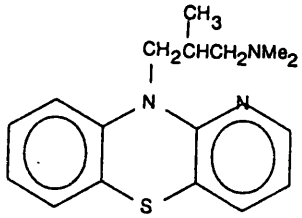
(b) Phenothiazines



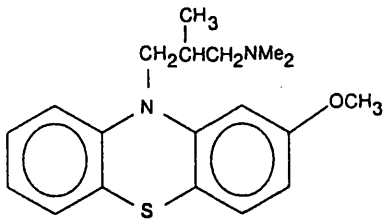
trimeprazine



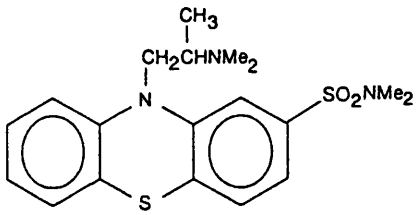
promethazine



isothipendyl

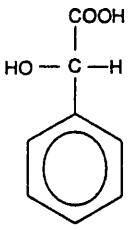


methotrimeprazine

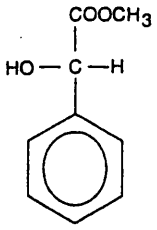


dimethothiazine

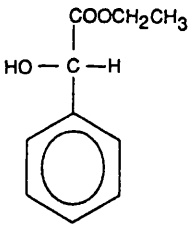
(c) Mandelic acids and related compounds



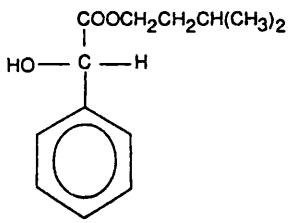
mandelic acid



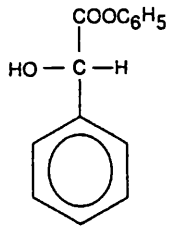
methyl mandelate



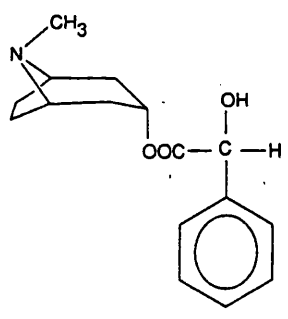
ethyl mandelate



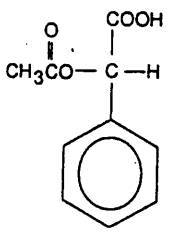
isoamyl mandelate



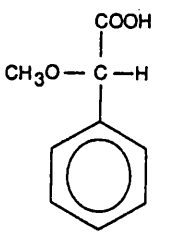
benzyl mandelate



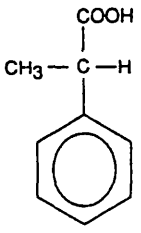
tropine mandelate



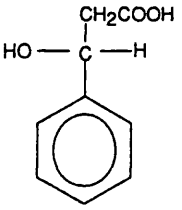
O-acetyl-mandelic acid



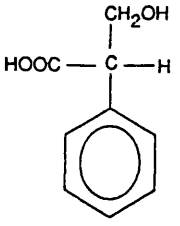
2-methoxy-phenylacetic acid



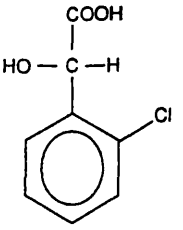
2-phenyl-propionic acid



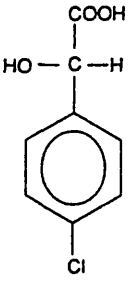
3-phenyl-lactic acid



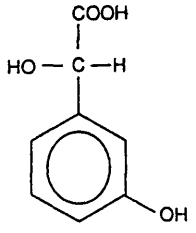
tropic acid



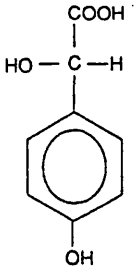
2-chloro-mandelic acid



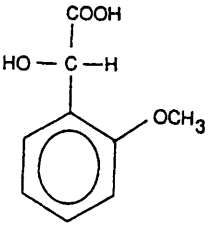
4-chloro-mandelic acid



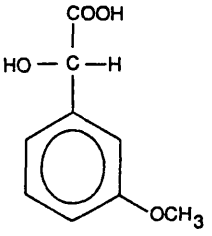
3-hydroxy-mandelic acid



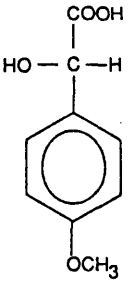
4-hydroxy-mandelic acid



2-methoxy-mandelic acid



3-methoxy-mandelic acid



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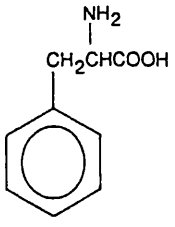

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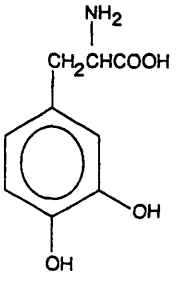
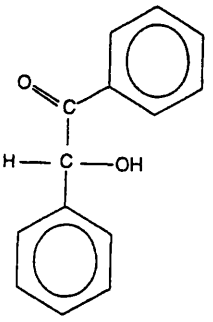
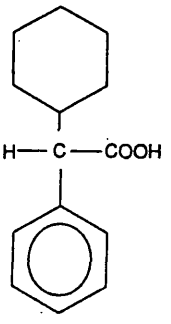


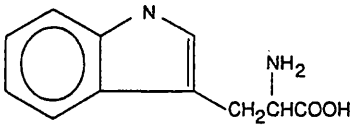
3,4-dihydroxy-phenylalanine



benzoin

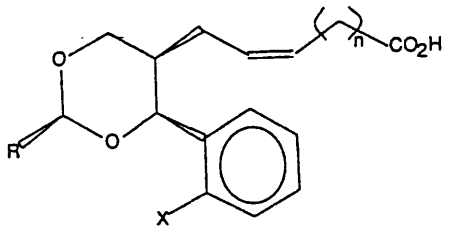


cyclohexyl-phenylacetic acid

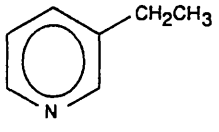


tryptophan

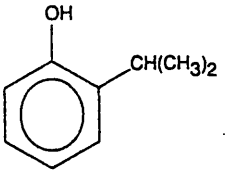
(d) Thromboxane antagonists and related model compounds



COMPOUND	R	X	n
TA1	3-pyridyl-CH_2-	-OH	2
TA2	3-pyridyl-	-OH	2
TA3	3-pyridyl-$C(CH_3)_2$-	-OH	2
TA4	3-pyridyl-CH=CH-	-OH	2
TA5	3-pyridyl-CH_2-$C(CH_3)_2$-	-OH	2
TA6	3-pyridyl-CH_2-CH_2-$C(CH_3)_2$-	-OH	2
TA7	N1-imidazoyl-CH_2-	-OH	2
TA8	Phenyl-O-$C(CH_3)_2$-	-OH	2
TA9	2-Chlorophenyl-	-OH	2
TA10	^{t}Butyl-	-OH	2
TA11	CF_3-	-OH	2
TA12	CF_3-	-OH	3
TA13	3-pyridyl-	-H	2
TA14	3-pyridyl-CH_2-	-H	3
TA15	3-pyridyl-	-OMe	2
TA16	3-pyridyl-CH_2-	-OMe	3
TA17	3-pyridyl-CH_2-CH_2-	-OMe	2

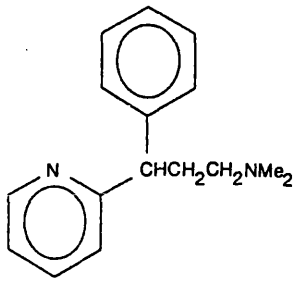


3-ethylpyridine

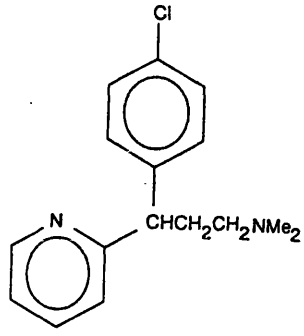


2-isopropylphenol

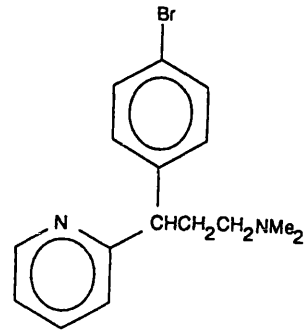
(e) Other chiral pharmaceuticals



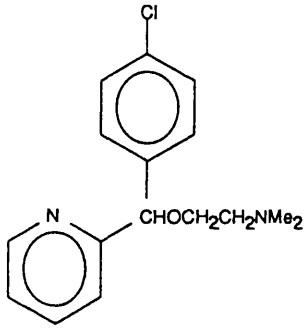
pheniramine



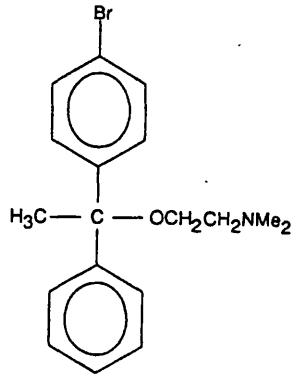
chlorpheniramine



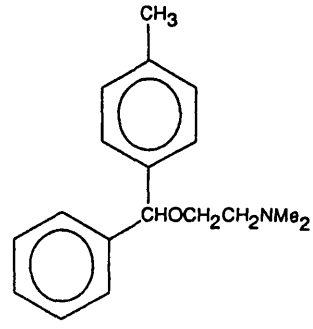
brompheniramine



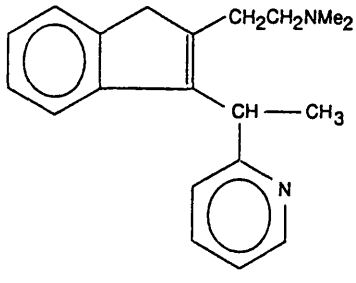
carbinoxamine



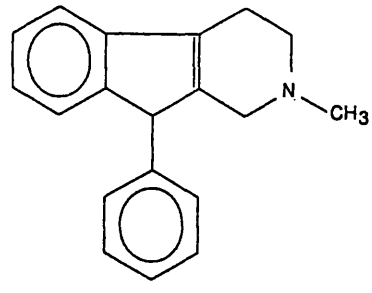
mebrophenydramine



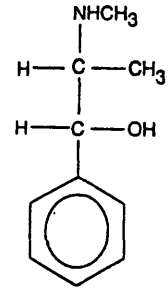
neobenodine



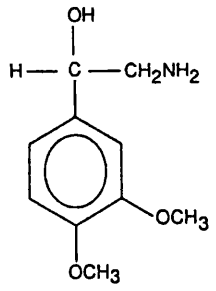
dimethindene



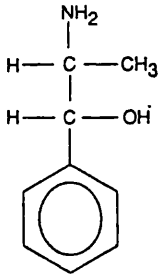
phenindamine



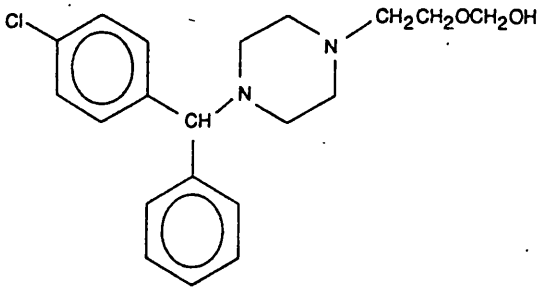
ephedrine



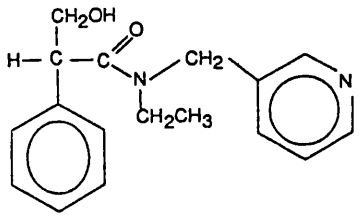
normetanephrine



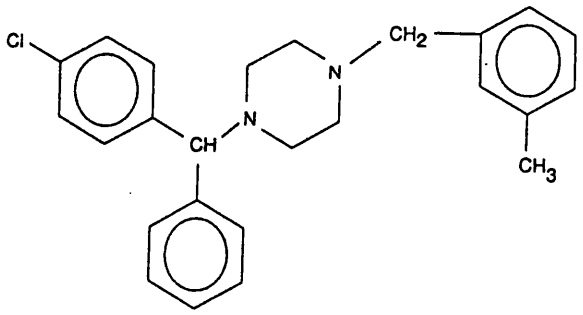
norephedrine



hydroxyzine



tropicamide



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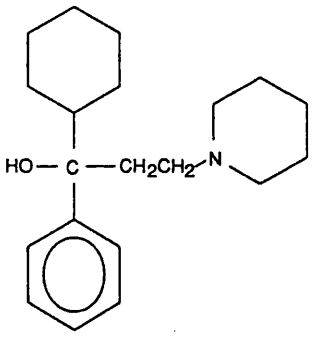
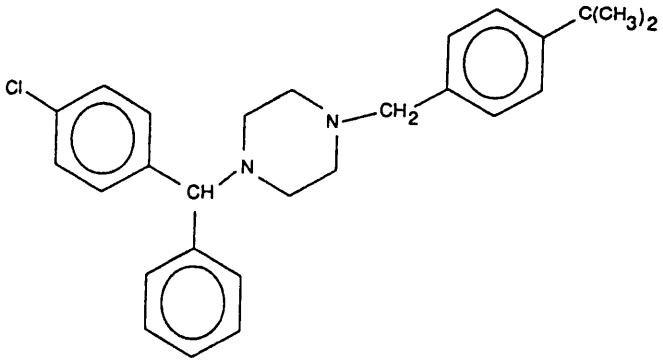


trihexyphenidyl



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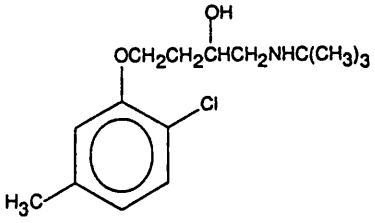
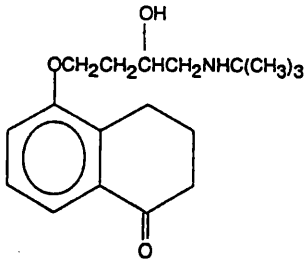


bupranolol



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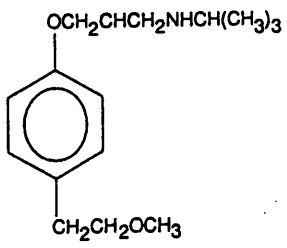
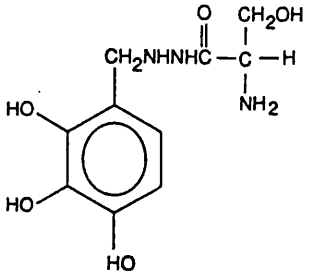
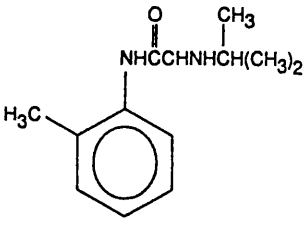


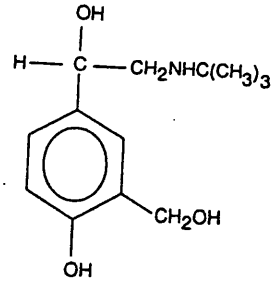
metoprolol



benserazide



prilocaine



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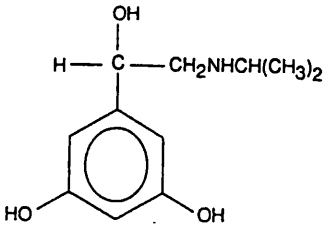
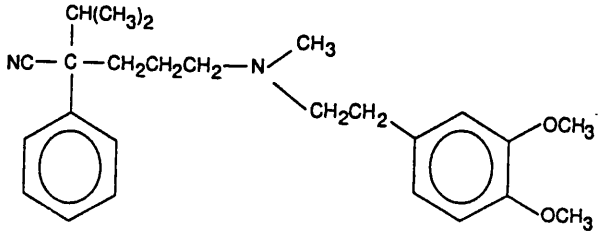
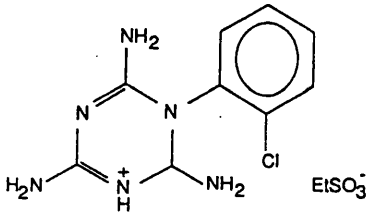


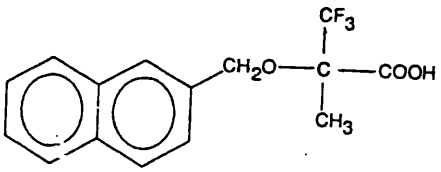
orciprenaline



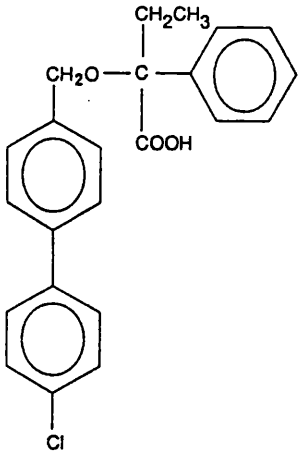
verapamil



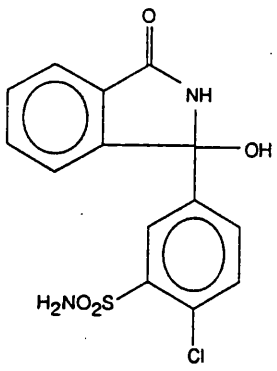
AM5(6)



ICI-2



ICI-1



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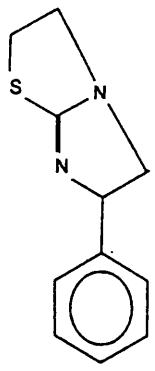

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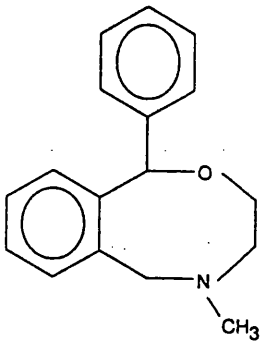
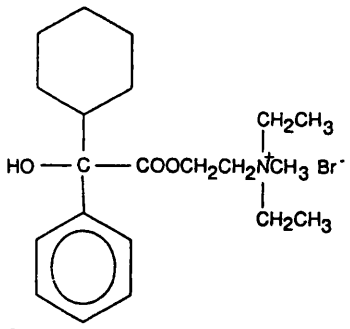
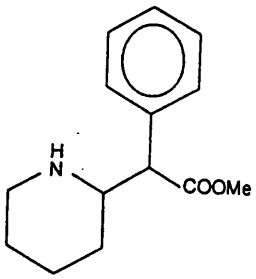


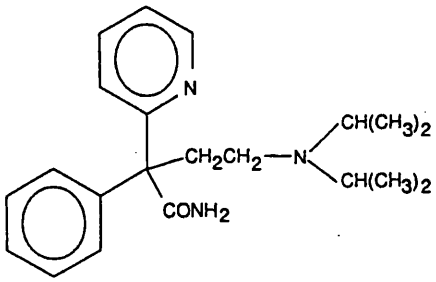
nefopam



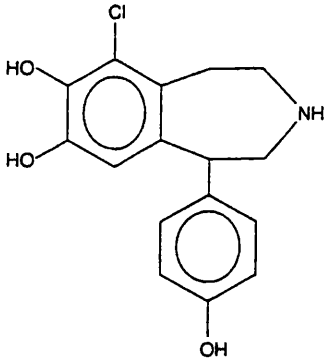
oxyphenonium bromide



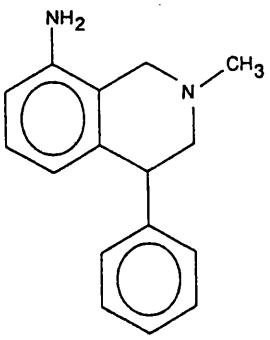
methyl phenidate



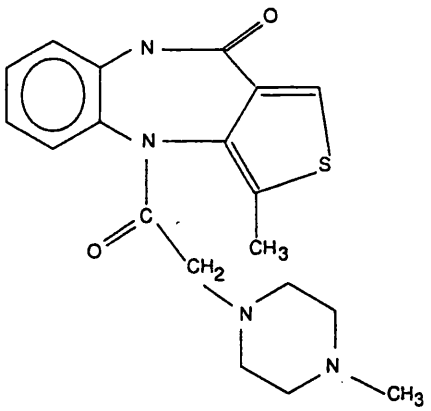
disopyramide



fenoldopam



nomifensine



telemzepine

2.1.7 HPLC packed columns and packing materials

The properties of the HPLC stationary phases employed during these studies are summarised in Table 2.1.

A 150 x 4.6mm column was packed with Hypersil 5µm CPS material (obtained from Shandon, Runcorn, Cheshire) using a Shandon column packer, operated by air pressure at 6000 psi, with the material slurried in acetone. A 100 x 4.6mm column was dry-packed with Lichrosorb Si60 12-20µm (obtained from BDH, Poole) for use as an in-line solvent saturation column. All other columns were obtained commercially.

Hypersil BDS C8 and Spherisorb C6 columns were packed by Capital HPLC (Edinburgh). Spherisorb S5CN 250 x 4.6mm and 250 x 10mm columns were obtained from Hichrom (Theale, Berks.). Other Spherisorb columns were obtained from Phase Separations (Deeside, Clwyd). Zorbax columns were obtained from Hichrom (Theale, Berkshire). SGE columns (packed with Spherisorb or Nucleosil materials as shown in table 2.1) were donated by SGE Ltd. (Milton Keynes). Semi-preparative (10mm i.d.) columns were packed by SGE Ltd. (Milton Keynes) using Lichroprep RP18 25-40µm (obtained from BDH, Poole) or Hamilton PRP-1 12-20µm (obtained from Hamilton, Reno, Nevada, USA). A Techsphere 3µm ODS column was donated by Perkin-Elmer (Beaconsfield, Bucks). A Cyclobond I 250 x 4.6mm column was obtained from Technicol (Stockport).

2.1.8 Other consumables

0.45µm cellulose nitrate (WCN), polyvinylenedifluoride (PVDF) and nylon (Nylaflo) membrane filters were obtained from FSA, Loughborough. 0.2µm Acrodisc filters were also obtained from FSA. Pre-cut lengths of microbore (0.02" i.d.) stainless steel tubing were obtained from HPLC Technology (Macclesfield, Cheshire).

Table 2.1. Properties of stationary phases employed in chromatographic studies.

column packing	description	s.a.	p.v.	m.p.d.	c.l.
		m^2/g	cm^3/g	nm	%
Hypersil CPS	cyanopropyl-silica	170	0.7	12	
Spherisorb S5CN	cyanopropyl-silica	220	0.8	8	3.5
Spherisorb A5Y C1	trimethyl-alumina			12	4
Spherisorb S5C6	hexyl-silica	220	0.8	8	4.6
Zorbax CN	cyanopropyl-silica			7	
Zorbax TMS	trimethyl-silica			7	
Techsphere ODS	3µm octadecyl-silica				
SGE-C8-8/5					
(Spherisorb C8)	octyl-silica	220	0.8	12	7
SGE-C8-30/5					
(Nucleosil 300C_8)	octyl-silica	100	0.8	30	2
Lichrosorb60 RP18	octadecyl-silica	315	0.8	10	17
Lichroprep RP18	25-40µm octadecyl-silica				
Zorbax PH	phenyl-silica			7	
Zorbax RX-C8	octyl-silica (base deactivated)			7	
Hypersil BDS C8	octyl-silica (base deactivated)			12	
Hamilton PRP-1	polystyrene-divinylbenzene (12-20µm)				
Cyclobond I	beta-cyclodextrin-silica				

Abbreviations:
s.a. = surface area
p.v. = pore volume
m.p.d. = mean pore diameter
c.l. = carbon loading, w/w

All packing materials listed have 5µm particle size unless otherwise stated.

NMR tubes (type 506-P) were obtained from Goss (Ingatestone, Essex).

2.2 Analytical HPLC studies

2.2.1 Equipment

Most of the analytical HPLC studies were carried out using a chromatographic system which consisted of an LDC Constametric III_G dual-piston HPLC pump, a Rheodyne 7125 manual sample injector fitted with a 5, 10, or 20µl loop, and a Spectromonitor III variable-wavelength UV detector connected to a Servogor 220 chart recorder. HPLC studies of thromboxane antagonists were carried out on a Varian LC5500 integrated liquid chromatograph equipped with a variable wavelength UV detector, an autosampler fitted with a 20µl loop, and a Varian data processing station.

Sample injections were made using a 50µl syringe (obtained from SGE Ltd., Milton Keynes).

A 100 x 4.6mm column, packed with LiChrosorb Si60 12-20µm, was placed in-line between the pump and the injector in order to pre-saturate the mobile phase with silica and hence protect the analytical column.

2.2.2 Preparation of mobile phases

Phosphate buffers were prepared by mixing 0.05M aq. NaH_2PO_4 with 0.05M aq. H_3PO_4 or 0.05M aq. Na_2HPO_4 to the required pH. Acetate buffers were prepared by a similar method, from aq. acetic acid and aq. sodium acetate. Triethylammonium acetate buffers were prepared by dissolving triethylamine at the required volume fraction in water and adding glacial acetic acid to the required pH. The pH of buffer solutions was checked using a Kent EIL 7020 pH meter, calibrated with aq. potassium hydrogen phthalate buffer at pH 4.

The aqueous portions of non-cyclodextrin-containing eluents were filtered through 0.45µm cellulose nitrate filters before addition of organic modifiers. Cyclodextrin-containing mobile phases were prepared by mixing buffer and organic modifier portions of the eluent, followed

by addition and dissolution of the cyclodextrin. Such mobile phases were filtered through 0.45μm polyvinylenedifluoride or nylon filters. All mobile phases were degassed by sparging with helium before use.

2.2.3 Operating conditions

The flow rates generally employed were 1ml/min. (through columns of 4.6mm internal diameter), 0.75ml/min. (through columns of 4mm internal diameter), 0.5ml/min (through columns of 3mm internal diameter), or 0.3ml/min (through columns of 2mm internal diameter).

Where practicable, column temperature was stabilised at 20-22°C, by immersion in a water bath containing a Grant thermostatic bath heater and BathCool cooling unit. Otherwise, HPLC experiments were carried out at ambient temperature.

Compounds were generally detected at their UV maxima (as reported in literature), in order to maximise sensitivity and selectivity. Where no literature value of UV maximum was available for a given compound, detection was effected at 254nm. Detector attenuation was adjusted as required to give reasonable peak heights in each case.

2.2.4 Solute preparation

Samples for injection at low column loading (0.1-1mg/ml) were dissolved in buffer, water, mobile phase, or dimethylsuphoxide-buffer (10:90, v/v), as required by their solubility characteristics. Where dissolution was incomplete, samples were filtered through 0.2μm Acrodisc filters before injection.

2.2.5 Determination of chromatographic parameters

(a) Capacity factor, k'

Capacity factors were determined from retention data according to equation 2.1.

$$k' = (t_R - t_0) / t_0 \qquad \text{(eqn. 2.1)},$$

where t_R and t_0 are the retention times of retained and unretained peaks, respectively.

t_0 values were determined by injection of water.

(b) Selectivity, α

The chromatographic selectivity between two enantiomeric peaks was calculated from their capacity factors according to equation 2.2.

$$\alpha = k'_1 / k'_2 \qquad \text{(eqn. 2.2)}.$$

(c) Column efficiency, $N_{w1/2}$

Column efficiency was calculated according to equation 2.3.

$$N_{w1/2} = 5.54 (t_R / w_{1/2})^2 \qquad \text{(eqn. 2.3)},$$

where $w_{1/2}$ is the peak width at half height.

For effective comparison of columns of differing lengths, L, efficiency values were often calculated on a "per metre" basis, where

$$N (m^{-1}) = N_{w1/2} / L \qquad \text{(eqn. 2.4)}$$

and L = column length in metres

(d) Resolution

The resolution of two solutes is conventionally expressed as R_S, given by equation 2.5.

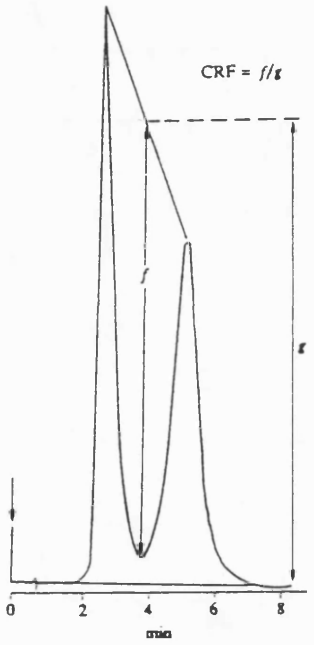
$$R_S = 2 (t_{R2} - t_{R1}) / (w_2 + w_1) \qquad \text{(eqn. 2.5)},$$

where w_1 and w_2 are the basal peak widths.

Basal peak width was detemined by extrapolation of the near-straight section of the peaks, in order to eliminate distortions due to peak tailing.

For poorly resolved and non-Gaussian peaks, a more useful measure of resolution was found to be Chromatographic Response Function (C.R.F.), as used by Clark and Mama (1989a) and defined in Figure 2.1.

Figure 2.1. Measurement of chromatographic response function (CRF). From Clark and Mama (1989a)



2.2.6 Definition of optical purity

Optical purity was measured in terms of enantiomeric excess (%e.e.), according to equation 2.6 (from Lindner and Pettersson (1985)).

%e.e. = ([enantiomer 1] - [enantiomer 2]) / ([enantiomer 1] + [enantiomer2]) x 100

(eqn. 2.6)

2.3. Semi-preparative resolution of trimeprazine enantiomers

2.3.1 Description of on-line recovery procedure

Trimeprazine enantiomers were separated on an SGE-100GLC4-C8-30/5 column (dimensions: 100mm length x 4mm i.d.), using a mobile phase consisting of acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 9 - 13.5mg/ml ß-cyclodextrin hydrate. 1mg injections of ±-trimeprazine hemi-(+)-tartrate were made onto the column using an LDC Promis 2 autosampler, at 100µl injection volume.

Following separation, the enantiomers were switched on to two "recovery" columns (Lichroprep RP18, 25-40µm particle size, 100 x 4.6mm dimensions) using the dual-valve integrated stream-switching (ISS) facility of the Promis 2 autosampler. Repeat injections (12 - 16) of racemate were made onto the separating column until the capacity of one of the recovery columns was reached. The recovery columns were then flushed with an aqueous-organic solvent mixture to remove cyclodextrin and buffer components. The trimeprazine fractions in each column were then eluted with a strong solvent (acetonitrile-TFA-water), and recovered as trifluoroacetate salts by evaporation of the solvent.

The switching valve arrangement is illustrated in Figure 2.2. The two Rheodyne 7000 valves that form the ISS facility on the Promis 2 allowed the switching of the solvent stream to recovery column 1 (for retention of the first enantiomer fraction), to column 2 (for retention of the second enantiomer fraction) or directly to waste (before and between the two peaks). A third Rheodyne 7000 valve, operated manually, allowed the the separation column to be by-passed during the flushing and elution stages of the procedure, thus reducing equilibration time prior to the next run.

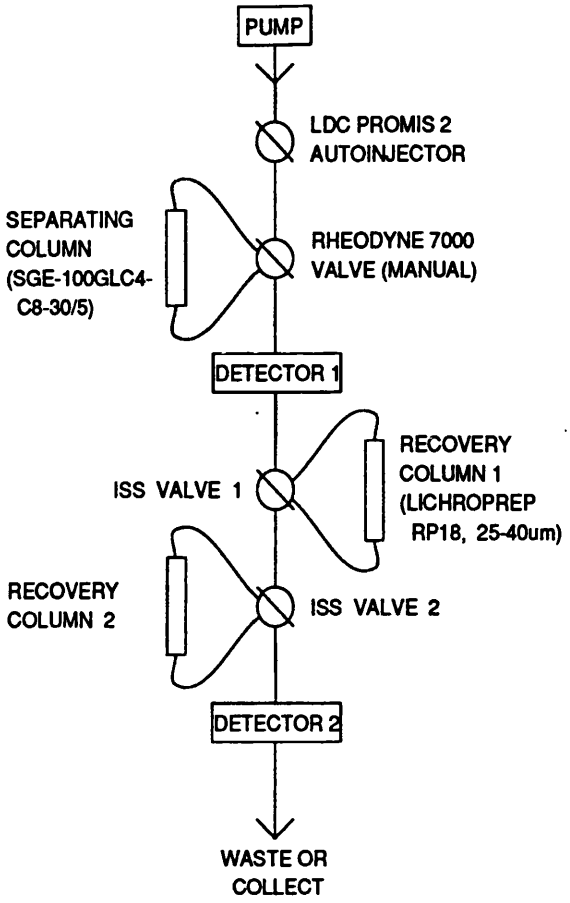


Figure 2.2. Column switching system used for on-line recovery of trimeprazine enantiomers following chiral separation using ß-cyclodextrin in the mobile phase.

Two UV detectors were placed in-line. The first (an LDC Spectromonitor 3000, set to 340nm 2AUFS) monitored the effluent from the separating column, and was used to determine the valve switching times. The second (a DuPont UV spectrophotometer with low-volume flow-cell, set to 302nm 1.28AUFS) monitored the effluent from the recovery system and was used to assess the progress of the loading, flushing and elution stages.

The eluted enantiomer fractions were assayed for recovery and optical purity as described below. Their chemical purity was assessed from their 270MHz ^{1}H-NMR spectra in D_2O.

2.3.2 Chiral assay of trimeprazine fractions from semi-preparative system.

The resolved trimeprazine enantiomers were assayed for recovery by re-injection into the chromatograph under similar conditions to those used for the semi-preparative resolution.

Calibration standards of ±-trimeprazine tartrate at 5, 50, and 500µg/ml in mobile phase were prepared, and assayed in duplicate to produce a calibration curve for each enantiomer. Levo-methotrimeprazine was used as internal standard. Peak height ratio calibration was carried out using a Spectra-Physics SP4270 computing integrator. Samples were prepared for analysis by mixing 500µl of standard solution or semi-preparative fraction with 500µl internal standard solution (50µg/ml), and 20µl injections were made in to the chromatograph using the Promis 2 autosamples.

The optical purity of each trimeprazine fraction was estimated by comparison of the levels of the two enantiomers measured by the above procedure. The eluates from the recovery columns collected during the "flushing" stage of the recovery procedure were also assayed by this method, to assess the loss of recovery sustained during this stage. Samples found to contain trimeprazine levels above the calibration range were diluted appropriately and re-assayed.

2.3.3 Optimisation of recovery procedure.

The capacity of recovery columns was estimated by experiments in which racemic trimeprazine was loaded onto the columns until "breakthrough" of UV-absorbing material was observed. The chromatograph employed in these studies consisted of an LDC constametric III_G pump, LDC Spectromonitor III UV recorder (set at 302nm, 0.2 AUFS to provide selective monitoring of trimeprazine), and a Servogor 220 chart recorder. The column was placed in a thermostatted water bath for studies into the effect of temperature on column capacity; ambient temperature was used in all other studies.

The recovery column under test was loaded with trimeprazine by pumping through it a solution consisting of acetonitrile - aq. triethylammonium acetate (0.88% TEA v/v, acetic acid to pH 4) (10:90 v/v) containing 13.5mg/ml ß-cyclodextrin hydrate and 0.1 - 0.2mg/ml

±-trimeprazine hemi-(+)-tartrate. The trimeprazine concentration was chosen so as to approximately simulate the trimeprazine concentration in the eluate from the semi-preparative separation described above. Column loading was ended when breakthrough of UV-absorbing material occurred, and the capacity estimated from the volume of solution passed up to this point.

Optimisation studies into the "flushing" procedure were carried out in a similar fashion to the above. In these studies the column was not necessarily loaded to its capacity. The flush solvent was passed though the column after loading, and the eluate collected for analysis. Eluates were assayed for trimeprazine in an achiral assay. This utilised an SGE-100GL4-C8-8/5 column, with a mobile phase consisting of acetonitrile- aq. triethylammonium acetate (1.3% TEA v/v, acetic acid to pH 4) (40:60, v/v) at a flow rate of 1ml/min. Standard solutions of trimeprazine tartrate at 5, 50 and 500µg/ml in mobile phase were prepared, and a calibration graph constructed using peak height ratios of trimeprazine to chlorpromazine (internal standard). Peak heights were measured using a Spectra-Physics SP4270 computing integrator. Samples and standards were prepared for analysis by adding 50µl of internal standard solution (500µg/ml) to a 500µl aliquot of the standard or unknown solution.

The flushing of triethylamine from a Hamilton PRP1 column (150x4.6mm) after equilibration of the phase with acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH4) containing 9mg/ml ß-cyclodextrin hydrate was investigated, using post-column ion-pair extraction detection to assay samples of the flush eluate (Roy and Jefferies (1990)). This assay utilised a chromatograph consisting of an SSI 220B pump, a Hypersil CPS (100x2.1mm) column, with injections made manually using a Rheodyne 7125 injector fitted with a 20µl loop. The mobile phase employed consisted of acetonitrile - 2-propanol - aq. NaH_2PO_4 (0.025M, adjusted to pH 4 with H_3PO_4) (7.5:7.5:85, v/v) at a flow rate of 0.2ml/min. Under these conditions, the triethylamine was unretained. On elution from the column, the eluate was mixed on-line with an aqueous solution containing 9,10-dimethoxyanthracene-

sulphonate (DAS) (1 x 10^{-4}M) at a flow rate of 0.4 ml/min, and subsequently with 1,2-dichloroethane at a flow rate of 0.5 ml/min. After passing through a stainless steel extraction coil (0.8mm i.d., 1.05m length, 50mm coil diameter), the organic and aqueous layers were separated using a dual channel sandwich-type phase separator (manufactured by SSI, State College, Penn., USA). The organic layer was then passed into an LDC Fluoromonitor detector (excitation filter 340-380nm, emmision filter 418-700nm, range 200), where the DAS-triethylamine ion-pairs were detected spectrofluorimetrically. Triethylamine concentrations were estimated by comparison of peak heights with previously injected standard solutions at 0, 0.008, and 0.0008% TEA, v/v.

2.3.4 Solvent extraction procedure for recovery of trimeprazine

The feasability of using solvent extraction to recover trimeprazine after semi-preparative resolution was investigated in an off-line experiment. A solution consisting of acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) containing 10mg/ml ß-cyclodextrin hydrate and 0.2mg/ml ±-trimeprazine hemi-(+)-tartrate was prepared. A 20ml portion of this solution was taken, and TFA was added to pH 1. The resulting precipitate was extracted into 4 x 5ml ether, and the organic phase dried over $MgSO_4$, before being evaporated to dryness, yielding non-crystalline trimeprazine trifluoroacetate.

A similar experiment was carried out on ±-trimeprazine, eluted from an RP18 recovery column in methanol after loading (6mg) as described above (2.3.3) and flushing the recovery column with 10ml of acetonitrile - water (10:90, v/v). The methanol was removed by evaporation, and the recovered trimeprazine reconstituted in water (10ml), and converted to the trifluoroacetate as described.

Trifluoroacetate salts prepared as above were converted to hydrochlorides by adding dry ethereal HCl to the trifluoroacetate in dry ether, causing precipitation of the hydrochloride.

2.4. Semi-preparative resolution of thromboxane antagonist enantiomers

2.4.1 Description of on-line recovery procedure

The procedure employed for resolution of the enantiomers of TA1 and TA12 was similar to that used for trimeprazine, with some differences in the instrumentation employed, as shown in Figure 2.3.

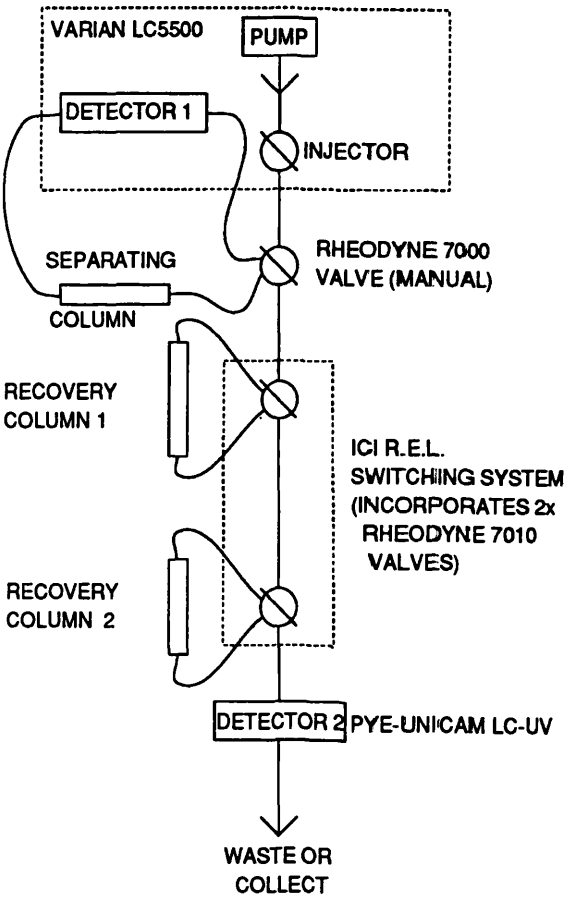


Figure 2.3. Instrumentation used to effect semi-preparative resolution of thromboxane antagonist enantiomers with on-line recovery.

Racemates were resolved on an SGE-100GLC4-C8-30/5 column (as before) using a mobile phase consisting of acetonitrile - aq. phosphate buffer (0.05M, pH 7) containing 20mg/ml ß-cyclodextrin hydrate. 1mg racemate injections were made manually onto the column, using a Rheodyne 7125 injector fitted with a 100µl injection loop. On-line recovery of the

enantiomers following separation was achieved using two Lichroprep RP18 columns as before. In this case, column switching was carried out using a purpose-built device, using timer control triggered by the injector (via an event marker output on the Varian LC5500 system). The Varian detector (detector 1) was set to 320nm, while the Pye-Unicam LC-UV detector (detector 2) was set to 280nm 1.28AUFS for more sensitive detection of the solutes.

Following elution from the recovery columns in methanol, a sample of each fraction was taken for optical purity analysis. The remainder of each fraction was evaporated to dryness under nitrogen and chemical purity determined from its 400MHz ^{1}H-NMR spectrum in CD_3OD.

2.4.2 Chiral assay of TA1 fractions after semi-preparative separation.

Resolved TA1 enantiomers were assayed for recovery by re-injection into the chromatograph under similar conditions to those employed for the semi-preparative resolution. Calibration standards of ±-TA1 at 50, 250 and 1250µg/ml in mobile phase were prepared, and injected in triplicate to produce a calibration graph for each enantiomer. Meta-nitroaniline was used as internal standard. Peak-height calibration was carried out by the Varian data system. Samples were prepared for analysis by mixing 200µl of standard solution or semi-preparative fraction with 50µl internal standard solution (0.1mg/ml) and 1ml mobile phase. 20µl injections were made using the Varian autosampler. Optical purity values were estimated as before, by comparison of the levels of each enantiomer found by the above procedure.

2.4.3 Chiral assay of TA12 fractions after semi-preparative separation.

Resolved TA12 enantiomers were assayed for recovery and optical purity on a Cyclobond I (250 x 4.6mm) column, using a mobile phase consisting of acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v) at a flow rate of 1ml/min. Calibration standards of ±-TA12 at 0.02, 0.06, 0.18, 0.54, and 1.62mg/ml in mobile phase were prepared, and injected in duplicate to

produce a calibration graph for each enantiomer. Meta-nitroaniline was used as internal standard. Peak-height ratio calibration was carried out by the Varian data system, except where the data system failed to recognise small peaks, in which case peak heights were measured manually and linear regression was carried out using a Casio FX-570c calculator. Samples were prepared for analysis by mixing 150µl standard solution or semi-prep fraction with 150µl internal standard solution (0.2mg/ml) and making up to 1.5ml with aq. phosphate buffer (0.05M, pH 7). 20µl injections were made using the Varian autosampler. The detection wavelength employed was 270nm, for maximum sensitivity. The quality of the assay was checked by assaying the injection solution from the semi-prep run (nominally 10mg/ml ±-TA12), diluted 1 in 20 to bring its concentration into the calibration range.

2.5. Semi-preparative resolution of brompheniramine enantiomers

2.5.1 Description of procedure

Brompheniramine enantiomers were separated on a Spherisorb S5CN column (dimensions: 250mm length x 10mm i.d.), using a mobile phase consisting of methanol - aq. triethylammonium acetate (0.85% TEA v/v, acetic acid to pH 4) (5:95, v/v) containing 12mg/ml ß-cyclodextrin hydrate. The instrumentation employed was similar to that shown in Figure 2.3, except that an LDC Constametric III_G pump and Spectromonitor III UV detector connected to a Servogor 220 chart recorder were used in place of the Varian LC5500 system. The detection conditions employed were 285nm 2AUFS (detector 1), 290nm 1.28AUFS (detector 2).

5mg injections of ±-brompheniramine maleate were made onto the column using a Rheodyne 7125 injector equipped with a 100µl loop. Following separation, the enantiomers were switched onto Hamilton PRP1 recovery columns (12-20µm particle size; 100 x 10mm dimensions) using the ICI REL valve switching system. 22-24 repeat injections of racemate were made, giving a total recovery column loading of about 60mg per enantiomer. The recovery columns were then flushed with 180ml of methanol - water (5:95, v/v); and the brompheniramine fractions were eluted with methanol.

A sample of each fraction was taken for subsequent analysis. The remainder of each fraction was evaporated to dryness, re-dissolved in mobile phase, and further purified by a second pass though the recovery system, under the conditions described above.

The eluted enantiomer fractions from each stage were assayed for recovery and optical purity by HPLC as described below. Their chemical purity was assessed from their 270MHz ^{1}H-NMR spectra in D_2O. Recovery was also checked by NMR, by addition of one mole equivalent of maleic acid as internal standard, and comparison of the resulting proton signal

integrals.

A sample of each fraction was converted to brompheniramine free base by solution in water, addition of excess conc. NaOH and extraction into ether. The ether extracts were evaporated to dryness, and the brompheniramine taken up in dimethylformamide. The optical rotation of the resulting solution was measured using an Optical Activity Ltd. AA-10 Polarimeter.

2.5.2 Chiral assay of brompheniramine fractions from semi-preparative system.

The resolved brompheniramine enantiomers were assayed for recovery and optical purity on a Spherisorb C6 column (150 x 3mm), using a mobile phase consisting of acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) containing 21mg/ml ß-cyclodextrin hydrate, at a flow rate of 1ml/min.

Calibration standards of ±-brompheniramine maleate at 0.01, 0.05 and 0.25mg/ml in mobile phase were prepared, and injected in triplicate to produce a calibration graph for each enantiomer. ±-pheniramine maleate was used as internal standard. Peak-height ratio calibration was carried out using a Spectra-Physics SP4270 computing integrator.

Samples were prepared for analysis by mixing 50µl standard solution or semi-prep fraction with 50µl internal standard solution (0.1mg/ml) and making up to 500µl with mobile phase. 10µl injections were made in partial loop-fill mode using a Rheodyne 7125 injector fitted with a 20µl loop, and a SGE 50µl guided plunger syringe. The detection wavelength employed was 254nm.

2.5.3. Optimisation of recovery procedure

Experiments were conducted in a similar manner to that described for trimeprazine in section 2.3.3. Brompheniramine was loaded onto the recovery column in a solution consisting of methanol - aq. triethylammonium acetate (0.85% TEA v/v, acetic acid to pH 4) (5:95, v/v)

containing 12mg/ml ß-cyclodextrin hydrate and 1mg/ml ±-brompheniramine maleate. The recovery column was flushed with methanol - water (5:95, v/v), and the brompheniramine eluted with methanol. Chemical purity and recovery were assessed by NMR, as described above. Comparison of the performances of Lichroprep RP18 and Hamilton PRP-1 as recovery column packings were made.

2.6. Determination of the solubility of ß-cyclodextrin in some aqueous-organic solvent mixtures

The solubility of ß-cyclodextrin in aqueous-organic solvent mixtures was determined by the gravimetric method of Jozwiakowski and Connors (1985). 350mg portions of ß-cyclodextrin hydrate were placed in 14ml screw-top vials containing 11ml of the solvent under test. The vials were sealed with PTFE tape. The vials were agitated in a thermostatted water bath at 20°C for 7 days, and extra portions of cyclodextrin added if neccessary during that time. The resulting saturated solutions were filtered through 0.2µm Acrodisc filters and a 10ml portion of each sample was evaporated to dryness overnight in at 60°C and < 10mbar pressure (in a vacuum oven). The concentration of the saturated solution was then determined by weighing the resulting anhydrous cyclodextrin.

2.7 NMR experiments

2.7.1. Spectrometer conditions

270 MHz ^{1}H-NMR spectra were recorded on a Jeol JNM-GX-270 instrument. 32K data points were used in acquiring spectra with widths of 3001.2 Hz at ambient temperature. A 5μs pulse corresponding to a tilt angle of 30° was employed with an aquisition time of 4.918 seconds and a 0.541 second pulse delay. Digital resolution was 0.18 Hz per point.

400 MHz ^{1}H-NMR spectra were recorded on a Jeol GX-400 instrument at the Department of Chemistry, University of Bristol. 32K data points were used in acquiring spectra with widths of 4000Hz. A pulse angle of 45° was employed with an aquisition time of 4.1 seconds and a pulse delay of 0.5 seconds.

400 MHz ^{1}H-NMR spectra of ±-TA1 were also recorded on a Bruker WM400 spectrometer. 16K data points were used in aquiring spectra with widths of 5000Hz. A 7μs pulse corresponding to a tilt angle of 45° was employed, with an aquisition time of 1.6 seconds. The probe temperature was 25°C. Digital resolution was 0.3Hz per point.

Solvent suppression was employed in recording spectra at 270MHz in H_2O, and in some 400MHz studies in D_2O. The method employed was homogated decoupling with the H_2O or HDO resonance irradiated using an irradation attenuation (IRATN) value of 120.

Spectra in D_2O were referenced internally to HDO (4.9ppm) or externally to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate, 0.00ppm). Samples were prepared in 5mm o.d. tubes.

The deuterium of the solvent was used to provide a lock signal in all experiments. 5% D_2O was added to H_2O in experiments involving solvent suppression for this purpose. The number of accumulations per spectrum was typically 32, but was increased to give additional

sensitivity where necessary.

2.7.2 Assignment of spectra

Assignments were made on the basis of expected chemical shifts and signal multiplicity, and/or by comparison with published data. Spin decoupling experiments were carried out where necessary. The 400MHz ^1H-^1H COSY spectrum of TA1 in CD_3OD was produced by Mr. D.F. Wood, School of Pharmacy and Pharmacology, University of Bath. nOe difference spectra were produced by irradiation of the signal of interest (at IRATN = 320) and subtraction of the resulting nOe spectrum from a control spectrum, produced by irradiation of a blank region of the spectrum.

2.7.3 Chiral discrimination trials

For chiral discrimination trials, a racemate concentration of 1.33×10^{-5}M was employed, with one mole equivalent of cyclodextrin added. Sodium salts of thromboxane antagonists and other acids were prepared *in situ* by addition of one mole equivalent of sodium carbonate.

2.7.4 Formation constant determination

Determination of cyclodextrin complex formation constants was carried out by addition of varying amounts of ß-cyclodextrin to a fixed amount of substrate, and measurement of the resulting changes in substrate chemical shift. Such experiments on single compounds were carried out according to the method of Smith *et al* (1989). Non-linear least-squares regression analysis of the data was carried out using MINSQ (MicroMath Software, Salt Lake City, Utah, USA) run in MS-DOS using an Acorn PC Emulator on an Archimedes 3000 micro-computer. Experiments to determine formation constants for both enantiomers of a substrate simultaneously were carried out by a similar method, and the system of equations outlined in Appendix 1 used to analyse the resulting data. Non-linear regression was again

carried out using MINSQ.

2.7.5 Job plot for ±-TA1 sodium salt / ß-cyclodextrin

A continuous variation (Job) plot for ±-TA1 sodium salt with ß-cyclodextrin was constructed according to the method of Greatbanks and Pickford (1987). The quantities (mg) of ±TA1, sodium carbonate and ß-cyclodextrin dissolved in D_2O (1ml) were 0/0/22.1, 1.81/0.50/16.98, 2.49/0.68/15.01, 3.88/1.07/11.44, 5.29/1.47/7.49, and 5.61/1.55/5.73.

2.7.6 ROESY experiment on ±-TA1/ß-cyclodextrin

A ROESY experiment was carried out on ±TA1 (3.88mg/ml, sodium salt) with ß-cyclodextrin (11.44mg/ml) in D_2O. A Bruker WM400 spectrometer was used, and the Z-Filter CAMEL method of Rance (1987) employed. This uses the decoupler to supply the spin-lock field. The problem of transmitter and decoupler being out-of-phase is overcome by the use of phase-cycled 90° decoupler pulses before and after the spin-lock field. A spin-lock (mixing) time of 0.8 seconds was employed, with a 90° (13.5µs) observe pulse and a relaxation delay of 5 seconds. 256 t_1 increments were used, with 64 accumulations per t_1 increment. Resulting 2D ROESY spectra were transformed using the Wutriche method, with HDO on resonance.

Chapter 3

Results: Analytical HPLC studies

3.1 Introduction

Cyclodextrins have found wide application as bonded phases and/or as eluent additives in HPLC, TLC and GC. Improved chromatographic separations for many species of interest have been achieved, particularly of positional, geometric and optical isomers which often provide difficult chromatographic problems. The separation mechanism in cyclodextrin chromatography has been shown to depend on the strength of interaction of the analytes with the cyclodextrin.

ß-cyclodextrin has found greater chromatographic use than the other native oligomers, due partly to its lower cost, but also its cavity size is optimal for inclusion of many solutes of interest.

3.1.1 Cyclodextrin stationary phases for liquid chromatography

Stable chromatographic stationary phases have been produced by modification or immobilisation of cyclodextrins. On such phases, solutes elute in order of increasing strength of interaction with the cyclodextrin.

The first cyclodextrin stationary phases to be reported were ß-cyclodextrin polymer gels, with mechanical rigidity conferred by cross-linking with epichlorohydrin. Wiedenhoff *et al* (1969) achieved column chromatographic separation of some benzoic acids by this approach. Harada *et al* (1978) reported resolution of ±-methyl mandelate into its enantiomers and partial resolution of ±-mandelic acid, ±-ethyl mandelate and ±-O-methyl mandelic acid using ß-cyclodextrin-epichlorohydrin (ß-CDE) as stationary phase. A ß-cyclodextrin polymer with poly-ether cross-links was used by Zsadon *et al* (1986) to resolve several indole alkaloid racemates.

HPLC stationary phases have been prepared by bonding cyclodextrins to microparticulate

silica. Kawaguchi *et al* (1983) produced such a phase by treating succinamidopropyl-silica with ethylenediamine-substituted ß-cyclodextrin. Fujimura *et al* (1983) produced similar stationary phases by bonding cyclodextrins to aminopropyl-silica. The chiral separations reported on these phases include those of cyclohexylphenylacetic and mandelic acids (Feitsma *et al* (1984)) and several mandelic acid esters (Fujimura *et al* (1986)).

An alternative cyclodextrin stationary phase has been produced and patented by Armstrong. In this phase, the cyclodextrin is linked to silica by a six to ten atom spacer containing no nitrogen (Ward and Armstrong (1986)). This linkage method overcomes the problems associated with previous phases, *i.e.* hydrolytic instability, low cyclodextrin loading, competing ion-exchange interactions due to the amine functionality in the spacer, and synthetic difficulties (Seeman *et al* (1988)). Armstrong's phases have been commercialised as the Cyclobond® range, and have been very widely used. Some of the chiral separations achieved on Cyclobond I (the ß-cyclodextrin phase) are illustrated in Table 3.1. Other chiral separations on this phase that have been reported include peptides (Florance *et al* (1987)), amino acid derivatives (Han and Armstrong (1987)), barbiturates and hydantoins (Berthod *et al* (1990), Maguire (1987)) metallocenes (Armstrong *et al* (1985)) and nicotine analogues (Seeman *et al* (1988)). Pharmaceutical applications, both in optical purity analysis and bio-fluid analysis have also been reported (Krstulovic *et al* (1988), Lee and Webb (1988), Walhagen *et al* (1989), McClanahan and Maguire (1986), Edholm *et al* (1988), Matsui and Sekiya (1989)).

More recently, a number of HPLC stationary phases incorporating derivatised cyclodextrins have been produced by Armstrong and his co-workers, using the same bonding chemistry as before (Astec (1991)). Hydroxypropyl, acetyl, toluyl, napthylisocyanate, and dimethyliso-cyanate derivatives of the three native cyclodextrins have been used to produce phases which dramatically extend the range of racemates resolvable by this approach. Several of these phases are used in normal-phase mode, with non-polar organic solvents as eluents. In

Table 3.1. Chiral separations reported using Cyclobond I (beta-cyclodextrin) HPLC columns

Solute	k'_1	α	R_s	ref.
ibuprofen	8.2	1.07	1.5	Geisslinger (1989)
ketoprofen	7.7	1.06	1.2	Berthod (1990)
mephobarbital	14.8	1.14	0.6	Ward (1986)
benzoin	3.2	1.08	1.0	Han (1988)
chlorthalidone	0.5	1.44	1.9	Berthod (1990)
benzyl mandelate	3.0	1.02	0.6	Han (1988)
mephenytoin	0.5	1.33	1.8	Armstrong (1986)
brompheniramine	7.8	1.14	1.5	Mercer (1989)
chlorpheniramine	5.9	1.07	1.5	Berthod (1990)
pheniramine	2.2	1.03	0.4	Mercer (1989)
carbinoxamine	3.0	1.07	0.8	Mercer (1989)
propanolol	2.8	1.04	1.4	Berthod (1990)
methyl phenidate	1.2	1.14	1.6	Berthod (1990)
nomifensine	2.0	1.35	3.0	Aboul-Enein (1988)
scopolamine	1.7	1.10	1.8	Armstrong (1987a)
cocaine	6.3	1.04	0.9	Armstrong (1987a)
atropine	6.8	1.04	0.6	Armstrong (1987a)
tropine mandelate	2.0	1.07	1.4	Armstrong (1987a)
nisoldipene	4.1	1.04	0.9	Armstrong (1986)
norgestrel	0.5	1.24	1.1	Armstrong (1986)

these cases, solute inclusion is hindered by preferential inclusion of eluent molecules. Retention and selectivity are thought to arise via interactions with the outside of the cyclodextrin molecule.

3.1.2. Factors affecting HPLC resolution on cyclodextrin stationary phases

Cyclodextrin stationary phases exhibit differing selectivity to conventional reversed phase materials, owing to the predominant retention mechanism being inclusion in the cyclodextrin cavity. Phases prepared from native cyclodextrins are always used with aqueous eluents containing organic modifiers (typically acetonitrile, methanol, ethanol, isopropanol). As in conventional RP-HPLC, an increase in the organic modifier concentration leads to a decrease in retention times and selectivity (Armstrong *et al* (1985a)). In cyclodextrin chromatography, this reflects the ability of the organic modifier to include in the cavity, reducing the binding of the solutes. Methanol forms a much less stable complex with ß-cyclodextrin than either ethanol or acetonitrile, and therefore causes less reduction in retention and selectivity at a given level in the mobile phase.

It is known from measurement of complex stability constants that an increase in temperature decreases the extent of complexation of a substrate (Hinze (1981)). Thus, selectivity on cyclodextrin bonded phases is generally reduced by increasing temperature. However, band spreading may be reduced at higher temperature due to improved retention kinetics. The overall effect of temperature on resolution is therefore not entirely predictable, with increases in resolution with temperature reported in some cases (Armstrong *et al* (1985a), but decreases in others (Isaaq *et al* (1987)).

Buffer components have also been shown to have an effect on resolution using cyclodextrin bonded phases. The use of triethylammonium acetate buffer is particularly beneficial, producing marked improvements in peak shapes (Ward and Armstrong (1986)). This is

thought to result from interactions between the triethylamine and cyclodextrin hydroxyls, reducing the incidence of strong interactions between solute amine groups and cyclodextrin hydroxyls, and thus improving the kinetics of the retention process

As might be expected, eluent pH has a marked effect on resolution of enantiomeric and other solutes using cyclodextrin phases. Thus, Feitsma *et al* (1985) reported a decrease in retention and enantioselectivity for mandelic acid on a ß-cyclodextrin phase on increasing pH from 4.2 to 6.5, reflecting weaker binding of the dissociated acid. For other acids, opposite effects were noted, indicating that the effect of dissociation on enantioselective binding to ß-cyclodextrin is not entirely predictable.

3.1.3 Cyclodextrin phases for GC and TLC

Smolkova-Keulemansova *et al* (1988) have prepared packed GC columns containing a phase consisting of ß-cyclodextrin and formamide deposited from aqueous solution onto an inert support. These columns were used to improve separations of positional isomers. A similar appproach has been taken by Sybilska and Jurczak (1989), who produced phases containing all three native cyclodextrins. The alpha-cyclodextrin phase was used to separate optically active hydrocarbons. Other workers (König (1989), Li *et al* (1990)) have prepared phases using mixtures of various alkylated and acetylated cyclodextrin derivatives, which are hydrophobic liquids, to coat capillary columns. A wide variety of racemic analytes have been resolved on these phases, which are more efficient than the earlier packed columns.

The preparation of a ß-cyclodextrin bonded RP-TLC phase has also been reported (Alak and Armstrong (1986)) . This was found to resolve the enantiomers of several analytes, giving analogous results to those obtained using the Cyclobond I phase in HPLC.

3.1.4 Cyclodextrins as mobile phase additives in liquid chromatography

Cyclodextrins may also be used as additives to chromatographic eluents. As described above for cyclodextrin stationary phases, separations are obtained due to the selectivity of the cyclodextrin's complexing ability, which is dependent on the size, shape and functionality of the solute molecules. In most cases, the addition of cyclodextrin causes a reduction in solute retention, by solubilising the solute in the mobile phase. The greatest reduction in retention will occur for the most highly complexed solute in a mixture. The elution order may therefore be reversed in a cyclodextrin mobile phase compared to that obtained using the analogous cyclodextrin stationary phase.

The first applications of cyclodextrins as eluent additives were in gel chromatography. Thus, Uekama *et al* (1977a) found that the addition of ß-cyclodextrin to an aqueous eluent caused reductions in the retention times of isomeric prostaglandins on an anion-exchange resin, and facilitated their separation. The first chiral separation by this approach was reported by Sato and Suzuki (1985), who separated the enantiomers of warfarin and of mandelic acid by chromatographing aqueous solutions of the racemates containing ß-cyclodextrin on Sephadex gel .

Sybilska and his co-workers have reported separations of a large range of positional, geometric and optical isomers using ß-cyclodextrin as an HPLC eluent additive. Some of the chiral separations reported are listed in Table 3.2. Many other groups have reported chiral separations using native cyclodextrins as eluent additives. Some of these are also listed in Table 3.2. Industrial applications of this approach have arisen, both in optical purity analysis (Berger *et al* (1989)) and in bio-analysis (Yoshikawa *et al* (1986)).

Table 3.2. Chiral separations reported using beta-cyclodextrin as a mobile phase additive in RP-HPLC

Solute	k'_1	α	R_s	ref.
mandelic acid	4.5	1.08	0.5	Debowski (1982, 1983)
3-hydroxymandelic acid	2.4	<1.08		Debowski (1983)
2-hydroxymandelic acid	4.6	<1.08		Debowski (1983)
2-methoxymandelic acid	7.1	<1.08		Debowski (1983)
2-chloromandelic acid	11.4	1.15	1.0	Debowski (1983)
O-acetylmandelic acid	9.1	1.04		Debowski (1982a)
phenylalanine	7.0	1.04		Debowski (1982a)
mephenytoin	8.4	1.15	1.5	Sybilska (1986)
methylphenobarbital	5.0	1.08	1.2	Sybilska (1986)
norgestrel	24.2	1.00	0.0	Gazdag (1986)
dansyl-phenylalanine	30.7	1.06	1.4	Takeuchi (1986)
pseudo-ephedrine	0.9	1.12	1.0	Mularz (1988)
nicotine	6.6	1.12	1.7	Armstrong (1987b)
trans-sobrerol	24.4	1.08	1.45	Italia (1990)
brompheniramine	5.1	1.13	1.7	Mularz (1988)
chlorpheniramine	8.3	1.11	1.4	Mularz (1988)
trimeprazine	7.9	1.31	4.1	Mularz (1988)
promethazine	17.9	1.12	1.9	Mularz (1988)
propanolol	22.6	1.04		Mularz (1988)
trihexyphenidyl	7.0	1.05		Mularz (1988)

3.1.5 Cyclodextrins as eluent additives in TLC and ITP.

The use of cyclodextrin mobile phases in TLC has been explored. Hinze and Armstrong (1980) reported improved resolution of substituted benzoic acids on a polyamide stationary phase using an aqueous eluent containing ß-cyclodextrin. Debowski *et al* (1985) used a similar approach with C18-silica reversed-phase TLC plates, and found strong correlations with results obtained using similar mobile phases in HPLC. Chiral separations of racemates such as labetalol, mephenytoin, metallocenes, nicotine derivatives, and amino acid derivatives were achieved under similar conditions by Armstrong *et al* (1988a). Improvements in separations could be achieved by the use of urea in the eluent to increase the solubility of ß-cyclodextrin (Hinze *et al* (1989)). ß-cyclodextrin has also been used as a leading electrolyte additive in isotachophoresis (ITP). By this means, separations of isomeric and enantiomeric compounds (including ±-pseudoephedrine and ±-promethazine) have been achieved (Jelinek *et al* (1989), Smolkova-Keulemansova (1988a)).

3.1.6 Theoretical aspects of cyclodextrin eluent HPLC

The theoretical basis of chromatographic separations using cyclodextrins as eluent additives has been widely discussed. Chromatographic data has been used to determine thermodynamic parameters for the cyclodextrin-solute complexes. The important equilibria thought to account for the retention properties of guest molecules in chromatographic systems using cyclodextrin-containing mobile phases are illustrated in Figure 3.1, as described by Uekama *et al* (1978b, 1978c).

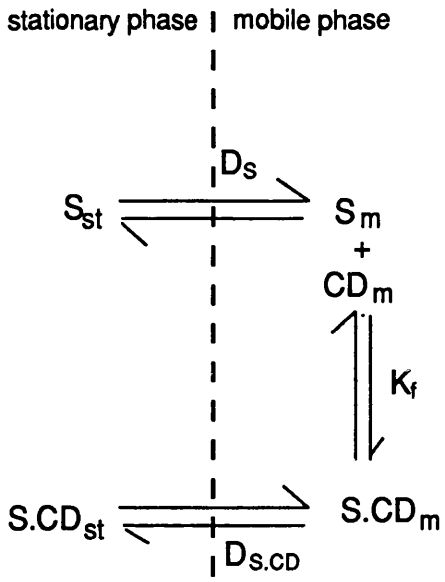


Figure 3.1. Equilibria responsible for effects of cyclodextrin in mobile phase in RP-HPLC (after Uekama *et al* (1978b))

The distribution ratios D_S, $D_{S.CD}$, and the 1:1 complex stability constant K_f are given by

$$D_S = [S]_{st}/[S]_m \quad \text{[eqn. 3.1]},$$

$$D_{S.CD} = [S.CD]_{st}/[S.CD]_m \quad \text{[eqn. 3.2]},$$

$$\text{and } K_f = [S.CD]_m/([S]_m.[CD]_m) \quad \text{[eqn. 3.3]},$$

where [S], [CD] and [S.CD] are the equilibrium concentrations of substrate (guest), cyclodextrin (host) and complex respectively, and the suffixes st and m refer to the stationary and mobile phases respectively.

The observed distribution ratio for the system, D_{obs}, is given by

$$D_{obs} = ([S]_{st}+[S.CD]_{st})/([S]_m+[S.CD]_m) \quad \text{[eqn. 3.4]}.$$

Combination of these equations yields

$$D_{obs} = (D_S + D_{S.CD}.K_f.[CD]_m)/(1 + K_f.[CD]_m) \quad \text{[eqn. 3.5]}.$$

The distribution ratios are related to retention times by

$$D_S = k'_S.\ V_m/V_{st} = (t_S-t_0/t_0).(V_m/V_{st}) \quad \text{[eqn. 3.6]},$$

$$D_{S.CD} = k'_{S.CD}\ .\ V_m/V_{st} = (t_{S.CD}-t_0/t_0).(V_m/V_{st}) \quad \text{[eqn. 3.7]},$$

$$\text{and } D_{obs} = k'_{obs}\ .\ V_m/V_{st} = (t_{obs}-t_0/t_0).(V_m/V_{st}) \quad \text{[eqn. 3.8]},$$

where t_0, t_S, $t_{S.CD}$ and t_{obs} are the retention times of an unretained solute, uncomplexed

substrate (*i.e.* at zero cyclodextrin concentration), complexed substrate (*i.e.* at infinite cyclodextrin concentration), and observed substrate peak at equilibrium, respectively. V_m/V_{st} is the phase volume ratio of the column.

Substitution then yields

$$t_{obs} = (t_S + t_{S.CD}.K_f.[CD]_m)/ (1 + K_f.[CD]_m) \qquad \text{[eqn. 3.9]},$$

which rearranges to

$$[CD]_m/(t_S-t_{obs}) = (1/t_S-t_{S.CD}).[CD]_m + (1/(K_f.(t_S-t_{S.CD})) \qquad \text{[eqn.3.10]}.$$

A plot of $[CD]_m/(t_S-t_{obs})$ against $[CD]_m$ thus gives a straight line of slope $1/(t_S-t_{S.CD})$ and intercept $1/(K_f.(t_S-t_{S.CD}))$, from which the stability constant and retention time of the complex can be determined.

A similar expression (equation 3.11) has been derived by Sybilska (1987).

$$k'_{obs} = ((k'_S-k'_{obs})/(K_f.[CD]_m)) + k'_{S.CD} \qquad \text{[eqn. 3.11]}.$$

In this case, a plot of k'_{obs} versus $(k'_S-k'_{obs})/[CD]_m$ gives a straight line of slope $1/K_f$ and intercept $k'_{S.CD}$.

These equations have been found to adequately describe the chromatographic process in most cases, and have given good agreement with K_f values determined by other means. A number of assumptions are inherent in the above treatment:

(1) that only one form of the guest is present,

(2) that the complex stoichiometry is 1:1,

(3) that the cyclodextrin does not change the properties of the stationary phase, *i.e.* $k'_{CD} = 0$, and

(4) that the equilibrium cyclodextrin concentration is the same as the initial concentration.

Modifications of Uekama's treatment have been proposed to deal with cases where the assumptions inherent therein may not be valid.

Sybilska *et al* (1982) have derived equation 3.12 for cases where ionised and unionised forms of an acid are present.

$$t_{obs} = (t_{HA} + (t_{A^-}.K_a/[H^+]) + (t_{HA.CD}.K_{f,HA}.[CD]_m) + (t_{A^-.CD}.K_{f,A^-}.[CD]_m.K_a/[H^+])) / (1 + K_a/[H^+] + K_{f,HA}.[CD]_m + (K_{f,A^-}.[CD]_m.K_a/[H^+]))$$ [eqn. 3.12],

where the subscripts HA and A^- refer to the unionised and ionised forms of the guest, respectively, and K_a is the acid dissociation constant.

Deviations from assumption (2) above have been considered by Debowski *et al* (1986), who derived equation 3.13 to include the possibility of 2:1 complexation.

$$k'_{obs} = (k'_S + (k'_{S.CD}.K_{f1}.[CD]_m) + (k'_{S(CD)2}.K_{f1}.K_{f2}.[CD]_m^2)) / (1 + K_{f1}.[CD]_m + K_{f1}.K_{f2}.[CD]_m^2)$$ [eqn. 3.13],

where $S(CD)_2$ refers to the 2:1 complex, K_{f1} applies to the first complexation step, and K_{f2} applies to the second.

They found that this treatment helped to explain certain anomalies in values obtained by equation 3.11 (particularly negative values of $k'_{S.CD}$), but that the K_{f1} values calculated from equation 3:13 did not differ by more than 10% from K_f values calculated using equation 3.11 for the cases studied.

Assumption (3) appears to be justified for the native cyclodextrins on reversed-phase silicas, since their capacity factors are reported to be very low (Fujimura *et al* (1986a)) owing to the hydrophilic nature of the outside of their cavities. Some authors have further simplified the equations by the additional assumption that complexes are also unretained (Fujimura *et al* (1986a), Cline Love and Arunyanart (1986), Mohseni and Hurtubise (1990)). Equations such as 3.14 have thus been derived.

$$1/k'_{obs} = 1/k'_S + K_f.[CD]_m/k'_S$$ [eqn. 3.14]

from which K_f is determined directly from the slope of a plot of k'_{obs} against $[CD]_m$.

Assumption (3) has been shown not to be valid for modified cyclodextrins and on highly hydrophobic phases (Zukowski *et al* (1988), Clark and Mama (1989a)), where retention of the cyclodextrin is greater.

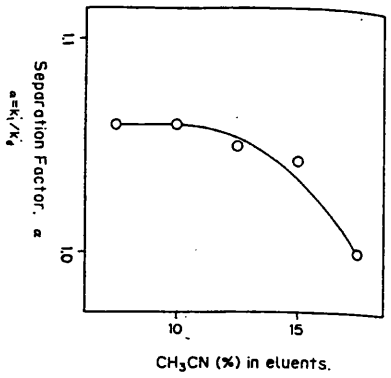
Assumption (4) is valid in the absence of organic modifiers, because the cyclodextrin is usually in large excess relative to the substrate, and so its concentration is not significantly reduced by substrate inclusion. However, the equilibrium cyclodextrin concentration may be reduced by inclusion of organic modifiers such as methanol or acetonitrile. This situation has been treated by Zukowski *et al* (1985), who proposed that the equilibrium cyclodextrin concentration in such a case might be given by

$$[CD]_m = [CD]_m^0/(K_{solv}[solv]_m^0 + 1) \qquad \text{[eqn. 3.15]},$$

where $[CD]_m^0$ and $[solv]_m^0$ are the initial molar concentrations of the cyclodextrin and the organic modifier, respectively, and K_{solv} is the formation constant of the 1:1 cyclodextrin-modifier complex.

The effect of organic modifiers on selectivity has been widely investigated. All authors have taken the view that low concentrations of organic modifiers are necessary for optimum selectivity when using cyclodextrin-containing eluents. This is illustrated in Figure 3.2, which shows the dramatic reduction in selectivity between dansyl-serine enantiomers with acetonitrile content of ß-cyclodextrin-containing eluents.

Figure 3.2. Effect of the mobile phase acetonitrile content on separation factor for dansyl-D,L-serine. Column: Hypersil ODS-3 (100x0.26mm); Mobile phase: acetonitrile-phosphate buffer (pH 5.0) containing 10mM ß-cyclodextrin. Flow rate: 2.1µl/min. From Takeuchi *et al* (1986).



It is also generally agreed that, of the commonly used organic modifiers, methanol has the lowest tendency to complex with ß-cyclodextrin. Matsui and Mochida (1979) estimated K_{solv} values for ß-cyclodextrin-alcohol systems from spectrophotometric measurements. They found values of 0.32 M^{-1} for methanol, 0.82 M^{-1} for ethanol, and higher values for higher alcohols. Fujimura *et al* (1986a) found that ß-cyclodextrin caused a larger change in retention of 1- and 2-naphthylamine isomers in methanol-containing mobile phases than in acetonitrile-containing eluents, and that tetrahydrofuran appeared to almost completely eliminate the effect of cyclodextrin on retention.

Equation 3.16 predicts that the apparent K_f value, K_f', determined from equation 3.11 will be related to the value determined from equation 3.15, K_f^0, by

$$K_f' = K_f^0/(K_{solv}.[solv]_m^0 + 1) \qquad \text{[eqn. 3.16]},$$

i.e. that apparent K_f should decrease with increasing organic modifier concentration. Whilst this is generally the case, Cline-Love and Arunyanart (1986) have reported anomalies. They found that apparent K_f values for a number of benzene derivatives increased on addition of 10% methanol (v/v) to aqueous mobile phases containing ß-cyclodextrin. They postulated that this effect was due to methanol inclusion aiding the substrate inclusion process, and their results indicate that Zukowski's treatment may be over-simplistic.

Another factor affecting choice of organic modifier is its influence on cyclodextrin solubility. Here, there is considerable disagreement in the literature, due to the lack of any detailed data regarding ß-cyclodextrin solubility in aqueous-organic solvent mixtures. Thus some authors (Sybilska (1987), Nobuhara *et al* (1983)) have preferred ethanol-buffer eluents; others have preferred acetonitrile (Mularz (1988), Takeuchi *et al* (1986)) or methanol (Gazdag *et al* (1986)) as organic modifier.

3.1.7 Modified cyclodextrins

A number of reports have appeared concerning the use of chemically modified cyclodextrins as mobile phase additives in TLC and HPLC. Tanaka *et al* (1985) reported the use of 2,6-di-O-methyl and 2,3,6-tri-O-methyl derivatives of alpha- and ß-cyclodextrin as mobile phase additives in RP-HPLC on a C18 column. Improved separations of some disubstituted benzenes compared to those achievable using unmodified cyclodextrins were obtained, particularly with the tri-methylated derivatives. There was strong evidence to suggest that significant interaction between the ODS stationary phase and the modified cyclodextrins was occurring. Thus, deviations from eqn. 3.14 were found for all solutes. Indeed, at low concentrations of the cyclodextrins, retention was found to increase relative to that seen in mobile phases containing no cyclodextrin. This was attributed to adsorption of the derivatives on the stationary phase, and was confirmed in RP-TLC experiments in which the derivatives were found to have significant retention on RP18 plates.

A number of other authors have reported results in accordance with the above. Thus, Zukowski *et al* (1986) reported reversal of elution order of enantiomers of some barbiturates on C18-silica on increasing the eluent concentration of dimethyl-ß-cyclodextrin. This was attributed to a change in retention mechanism from predominant interaction of the solutes with cyclodextrin adsorbed on the stationary phase (at low eluent additive concentrations) to predominant interaction of the solutes with cyclodextrin in the mobile phase (at high eluent

additive concentrations). The adsorption of trimethyl-ß-cyclodextrin on the ODS phase was such that interaction with adsorbed cyclodextrin on the stationary phase was found to be the dominant mechanism of selectivity at all additive concentrations (Zukowski *et al* (1988)). In this case, the inherent enantioselectivity of the additive towards the enantiomers of the barbiturates studied was found to be opposite to that observed with underivatised ß-cyclodextrin. The generation of "dynamic chiral stationary phases" by adsorption of such derivatised cyclodextrins onto C18-silica has been studied in more detail (Zukowski and Nowakowski (1989)). Improved chiral recognition and higher column efficiencies were found for a number of racemates, compared to those observed using underivatised cyclodextrin in mobile phase.

Clark (1989, 1989a) has described resolution of enantiomers on porous graphitic carbon (PGC) stationary phase using dimethyl-ß-cyclodextrin as a mobile phase additive. On this material, dynamic CSP generation was found to be the mechanism responsible for resolution at all additive concentrations. Thus, the retention and selectivity for chlorpheniramine enantiomers increased on increasing the additive concentration from 15 to 25mM. The other principal advantage of the use of derivatised cyclodextrins identified by Clark was their increased solubility in aqueous-organic solvent mixtures, allowing the use of a much wider range of solvent compositions.

Partially substituted hydroxyalkyl-cyclodextrins have also been used as chromatographic eluent additives. These have even greater solubility than the methylated derivatives, although the fact that the steric bulk of the substituents is thought to interfere with chiral recognition, and this has limited their utility. Armstrong *et al* (1988b) reported improved TLC separation of enantiomers using hydroxypropyl- and hydroxyethyl-ß-cyclodextrins with various degrees of substitution. While the derivatives with high degrees of substitution were found to have highest solubility in the eluents, chiral recognition was less with these derivatives. This was attributed to the loss of hydroxyl binding sites around the rim of the

cyclodextrin cavity. The high viscosity of eluents containing high additive levels was found to lead to increased development times. Clark and Mama (1989a) found that derivatised cyclodextrins of this type did not give improved separations of the solutes studied on PGC.

The addition of dimethyl- and trimethyl-cyclodextrins to electrolytes in isotachophoresis (Snopek *et al* (1988)) and capillary zone electrophoresis (Fanali and Sinibaldi (1989)) has been reported. Resolution of ephedrine enantiomers, which was unachievable using unmodified ß-cyclodextrin, was achieved using dimethyl-ß-cyclodextrin in both techniques.

3.2 Tetrahydroisoquinolines

3.2.1 ß-cyclodextrin mobile phases

A series of tetrahydroisoquinolines was selected for study with cyclodextrin-containing eluents because several of them exhibited chiral discrimination in NMR experiments with cyclodextrins. None of the seven tetrahydroisoquinolines investigated could be resolved into their enantiomers using ß-cyclodextrin-containing mobile phases, although complexation was evidenced by reduction in retention on addition of the cyclodextrin, as illustrated in Table 3.3. Table 3.4 gives typical data obtained for these solutes with a ß-cyclodextrin containing eluent. Increasing pH from 4 to 7.4 was seen to increase retention (due to partial suppression of amine protonation increasing the hydrophobic character of the solutes) but did not lead to resolution of enantiomers.

Table 3.3. Effect of beta-cyclodextrin in the mobile phase on retention of tetrahydroisoquinolines

Solute	Mobile phase	k'
TQ1	A (no CD)	8.6
	B (+ CD)	6.9
TQ2	A (no CD)	4.9
	B (+ CD)	4.5

Column: Hypersil CPS 100 x 4.6mm

Mobile phase A: Ethanol - aqueous diethylammonium acetate (0.5% DEA v/v, acetic acid to pH 4.7) (12:88 v/v)

Mobile phase B: As mobile phase A with the addition of 20mg/ml beta-cyclodextrin hydrate.

Table 3.4. Retention of racemic tetrahydroisoquinolines using (a) beta-cyclodextrin mobile phase and (b) beta-cyclodextrin stationary phase

Solute	Retention (k')	
	(a) BetaCD eluent	(b) BetaCD bonded phase
TQ1	1.5	2.1
TQ2	1.3	1.1
TQ3	1.6	1.9
TQ4	2.4	3.8
TQ5	1.8	3.0
TQ6	2.1	2.1
TQ7	3.0	

Conditions: (a) Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: acetonitrile - aqueous triethylammonium acetate (0.8% TEA v/v,acetic acid to pH 4.0) (10:90 v/v) containing 20mg/ml beta-cyclodextrin hydrate.

(b) Column; Cyclobond I (250 x 4.6mm); Mobile phase; methanol - aqueous triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4.0) (5:95 v/v)

3.2.2 Cyclobond I data

The tetrahydroisoquinolines were also unresolved on a Cyclobond I column. Typical data is shown in Table 3.4. The solutes were not strongly retained, even with only 5% methanol in the mobile phase, indicating that inclusion interactions were weak. Increasing pH from 4 to 7 increased retention but did not lead to chiral resolution.

3.2.3 Comparison of Cyclobond I and ß-cyclodextrin eluent data.

Comparison of the capacity factor (k') values for the two systems given in Table 3.4 was

carried out, and a statistically significant positive correlation obtained indicating that hydrophobic interactions with the stationary phase probably determine the retention order in both systems, and that inclusion interactions in the mobile phase do not substantially influence retention order.

3.3 Phenothiazines

3.3.1 ß-cyclodextrin mobile phases

Resolution of trimeprazine, promethazine and methotrimeprazine enantiomers could be achieved using ß-cyclodextrin mobile phases. Resolution of isothipendyl and dimethothiazine enantiomers could not be achieved. Typical data is shown in Table 3.5.

Table 3.5. Resolution of racemic phenothiazines using (a) beta-cyclodextrin mobile phase and (b) beta-cyclodextrin stationary phase

Solute	(a) BetaCD eluent			(b) BetaCD bonded phase		
	k'_1	α	R_S	k'_1	α	R_S
isothipendyl	3.6	not resolved		2.3	not resolved	
promethazine	1.3	1.06	0.5	3.8	not resolved	
dimethothiazine	4.7	not resolved		3.3	not resolved	
trimeprazine	0.5	1.22	0.8	10.0	1.11	1.0
methotrimeprazine	0.3	1.2	0.6	27.1	1.13	1.5

Conditions: (a) Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: acetonitrile - aqueous triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4.0) (10:90 v/v) containing 20mg/ml beta-cyclodextrin hydrate

(b) Column: Cyclobond I (250 x 4.6mm); Mobile phase: acetonitrile - aqueous triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4.0) (10:90 v/v)

Addition of ß-cyclodextrin to mobile phases caused a significant decrease in retention for the racemates that were resolved, but had little effect on the retention of isothipendyl and dimethothiazine, indicating that these substrates may interact with ß-cyclodextrin weakly or not at all. Increasing mobile phase pH from 4 to 7.4 was found to increase retention times by a factor of about 2 in all cases, but did not have any apparent effect on chromatographic enantioselectivity.

3.3.2 Cyclobond I data

As shown in Table 3.5, only trimeprazine and methotrimeprazine of the five chiral phenothiazines studied could be resolved on a Cyclobond I column. Retention times were much longer for these solutes than for the unresolved cases, indicating stronger inclusion interactions.

3.3.3 Comparison of Cyclobond I and ß-cyclodextrin eluent data

The elution order of the phenothiazines investigated was seen to be reversed using the Cyclobond I column compared to that seen using ß-cyclodextrin eluents. The elution order obtained using ß-cyclodextrin eluents was also opposite to that observed on the same (achiral) stationary phase in the absence of the cyclodextrin. Inclusion interactions are therefore prominent in determining the retention order in both the cyclodextrin eluent and cyclodextrin stationary phase systems.

Since the retention order on Cyclobond I was the same as that in the achiral reversed-phase system, it seems that hydrophobicity largely determines the strength of interaction with the cyclodextrin. This observation is in accordance with that of Otagiri *et al* (1975), who found a positive correlation between hydrophobicity (log P values) and complex stability (K_f) for a series of phenothiazines.

3.3.4 Correlation of enantioselectivity with structure

In this series, there appears to be a strong correlation between hydrophobicity, strength of complexation and chromatographic enantioselectivity. Thus, methotrimeprazine, which is most retained in the absence of ß-cyclodextrin, exhibits the largest change in retention on addition of ß-cyclodextrin and also the largest enantioselectivity. Isothipendyl is the most polar of the solutes, due to partial ionisation of its pyridyl group at pH 4, and showed no evidence here of interaction with ß-cyclodextrin. Dimethothiazine, however, was an anomalous case, being more retained than promethazine in the absence of ß-cyclodextrin but not interacting strongly with the cyclodextrin. In this case, complexation may be sterically hindered by the sulphonamide group on the phenothiazine nucleus.

3.4 Mandelic acids and related compounds

3.4.1 ß-cyclodextrin mobile phases

A series of compounds related to mandelic acid were investigated. These were mandelic acid itself; 2- and 4-chloromandelic acids; 2-, 3-, and 4-methoxymandelic acids; 3- and 4-hydroxymandelic acids; tropic, 3-phenyllactic, 2-methoxyphenylacetic, and O-acetylmandelic acids. The solutes were largely unretained and unresolved at pH 7, even on an ODS column. In several cases, samples of the two enantiomers were available. Replicate injections were made, and an accurate assessment of chromatographic enantioselectivity made. In all these cases, selectivity was not significantly different from unity.

Mandelic acid itself was found to exhibit some resolution under similar conditions to those employed by Sybilska (1987), *i.e.* using an eluent consisting of aq. phosphate buffer (0.05M, pH 2.1) containing 17mg/ml beta-cyclodextrin hydrate on a Hypersil ODS column (250 x 4.6mm). Other compounds in the series were not resolved under these conditions, in contrast to Sybilska's results.

Of particular note is the failure to resolve the enantiomers of 2-chloromandelic acid. Sybilska (1987)) reported that this racemate was well resolved (α = 1.8) under similar conditions to those used in this study.

The methyl, ethyl, benzyl and isoamyl esters of mandelic acid were also investigated. Retention was higher than for the ionised acids, due to greater hydrophobicity, but no resolution was observed.

3.4.2 Cyclobond I data

All the mandelic acids studied were found to be unresolved on a Cyclobond I column, both at pH 7 and at pH 2.5. At pH 7, all 12 compounds were unretained using an eluent containing 10% methanol (v/v) in phosphate buffer, indicating that inclusion interactions with the ionised soluted were weak. At pH 2.5, higher retention was observed in some cases, but no resolution.

3.5 Thromboxane antagonists

3.5.1 ß-cyclodextrin mobile phases

The enantiomers of fourteen of the seventeen thromboxane antagonists investigated were resolved by ß-cyclodextrin-containing mobile phases, as shown in Table 3.6. (The structures of these compunds are shown on p.55). In some cases, chromatographic selectivity was very high. Baseline resolution of enantiomers was achieved in eight cases. In two cases (TA6 and TA17), resolution was only observed when mobile phases containing urea were employed. In such eluents, a higher concentration of ß-cyclodextrin than is normally possible could be employed.

Table 3.6. Resolution of racemic thromboxane antagonists using (a) beta-cyclodextrin in mobile phase (b) a beta-cyclodextrin stationary phase.

Solute	(a) BetaCD eluent				(b) BetaCD bonded phase		
	k'_1	α	R_S	conditions	k'_1	α	R_S
TA1	7.4	1.62	5.2	(a_1)	7.0	1.35	3.1
TA2	6.0	1.13	1.0	(a_2)	7.3	1.10	1.2
TA3	33.5	1.58	5.7	(a_1)	9.6	1.24	2.2
TA4	28.9	1.21	2.1	(a_1)	10.6	1.11	1.4
TA5	56.0	not resolved		(a_1)	11.8	1.06	0.6
TA6	96.0	not resolved		(a_1)	33.7	1.08	0.8
	58.3	1.11	0.8	(a_2)			
TA7	5.6	1.21	0.9	(a_1)	4.1	1.10	1.1
TA8	>100	not eluted		(a_1)	11.8	1.06	0.6
	0.8	not resolved		(a_3)			
TA9	18.6	1.19	1.5	(a_1)	10.0	1.11	0.8
TA10	11.4	1.06	0.7	(a_1)	18.0	1.06	0.8
	15.8	1.11	1.0	(a_4)			
TA11	9.1	1.22	2.3	(a_1)	4.0	1.23	2.4
TA12	20.2	1.22	2.3	(a_1)	5.9	1.16	1.7
TA13	5.7	1.28	2.1	(a_1)	14.2	1.25	2.5
TA14	10.5	1.66	4.8	(a_1)	30.1	1.34	4.8
TA15	45.9	not resolved		(a_1)	8.4	not resolved	
TA16	>100	not eluted		(a_1)	11.4	1.07	0.7
	1.6	not resolved		(a_3)			
TA17	>100	not eluted		(a_1)	15.9	1.11	1.0
	35.1	1.06	0.6	(a_2)			

Table 3.6 (contd.)

Conditions: (a_1) Column: SGE100GL4-C8-30/5; Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7.0) (10:90 v/v) containing 20mg/ml beta-cyclodextrin hydrate.

(a_2) Column: SGE100GL4-C8-30/5; Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7.0) (10:90, v/v) containing 120mg/ml urea and 40mg/ml beta-cyclodextrin hydrate.

(a_3) Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: as a_1.

(a_4) Column: SGE100GLC4-C8-8/5; Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v) containing 240mg/ml urea and 100mg/ml beta-cyclodextrin hydrate.

(b) Column: Cyclobond I (250 x 4.6mm); Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7.0) (10:90, v/v)

The effect of pH on retention and selectivity was investigated, with TA1 (R=CH_2-pyr) and TA12 (R=CF_3) used as examples. The variation in retention with pH in the absence of ß-cyclodextrin for these two compounds is illustrated in Figure 3.3. A decrease in retention with increasing pH was observed for TA12, reflecting an increase in carboxyl group ionisation. The pH-retention relationship for TA1 was more complex, reflecting deprotonation of the pyridyl group (pKa about 5) and carboxyl group ionisation (pKa about 4) as pH was increased.

The pH-retention relationships of these compounds proved to be very similar to Figure 3.3 when using ß-cyclodextrin-containing eluents. This indicates that hydrophobic effects are still important in determining retention, even in the presence of inclusion interactions in the mobile phase. The variation of chromatographic enantioselectivity with pH for these compounds in the presence of ß-cyclodextrin is illustrated in Figure 3.4.

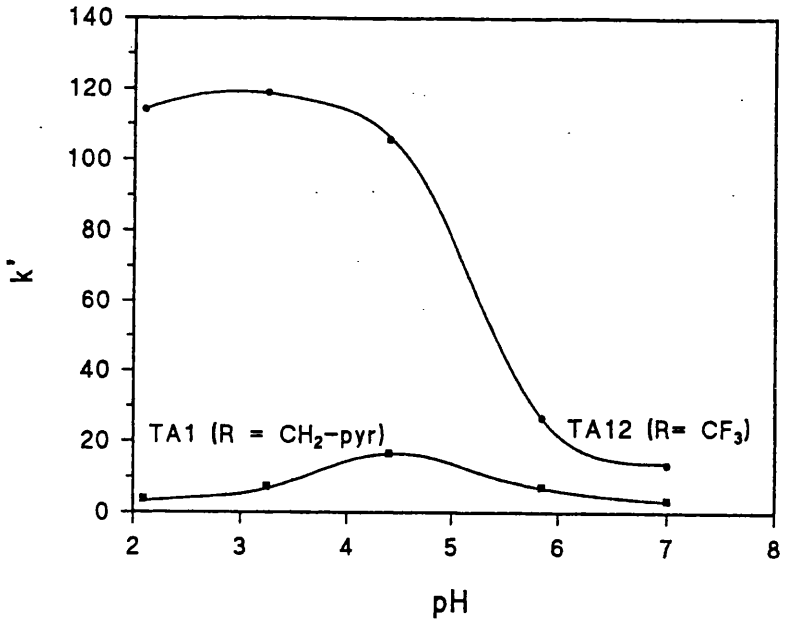


Figure 3.3. Variation of k' with pH for thromboxane antagonists in the absence of ß-cyclodextrin. Column: SGE-100GL4-C8-30/5; Mobile phase: acetonitrile - aq. phosphate buffer (0.05M) (22:78, v/v).

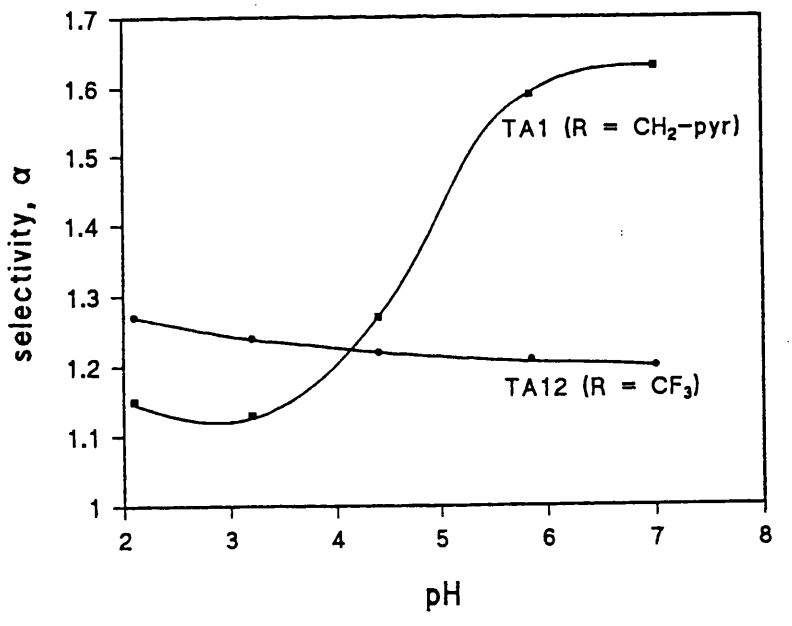


Figure 3.4. Variation of enantioselectivity with pH for thromboxane antagonists. Column: SGE-100GL4-C8-30/5. Mobile phase: acetonitrile - aq. phosphate buffer (0.05M) (10:90, v/v) containing 20mg/ml ß-cyclodextrin hydrate.

For TA12, a slight increase in selectivity was observed as pH was decreased, reflecting stronger complexation of the non-ionised solute at low pH. That this change is small probably reflects the length of the alkyl chain between the ionisable carboxyl group and the chiral centres and/or the the included portion of the molecule. The pH-selectivity relationship for TA1 was very different from that of TA12. In this case, a marked decrease in selectivity was observed as pH was decreased. This suggests that the ionisation of the pyridyl group of TA1 at low pH markedly disfavours enantioselective inclusion. This group is therefore seen to have a large effect on the inclusion of this solute, and its presence may account for the fact that at pH 7 TA1 is resolved much more effectively than TA12 by ß-cyclodextrin. Other pyridine-containing compounds in the series were seen to exhibit similar behaviour to TA1 on changing eluent pH.

3.5.2 Cyclobond I data

Using a Cyclobond I column, all but one of the thromboxane antagonists studied were resolved into their enantiomers, using the conditions shown in Table 3.6. Six of the seventeen racemates were baseline resolved under the conditions employed.

3.5.3 Comparison between Cyclobond I and ß-cyclodextrin eluent data

Direct comparison was made between the two systems by employing the same mobile phase (acetonitrile: 0.05M aqueous phosphate buffer (pH 7), 10:90 v/v) in both cases, with the addition of 20mg/ml ß-cyclodextrin to the mobile phase used with the achiral stationary phase.

A significant positive correlation, though far from linear, was found between the selectivities obtained using the two systems, indicative of similarities between the resolution-producing interactions. In general, higher selectivity was obtained using the ß-cyclodextrin eluent.

3.5.4 Correlation of enantioselectivity with structure

It has already been noted that ionisation of the pyridyl group in compounds such as TA1 has a marked effect on the chromatographic enantioselectivity induced by ß-cyclodextrin. However, the presence of this group *per se* is not necessary for enantioselective inclusion (*viz.* the selectivity observed for compounds TA7 to TA12). At least one other group in these structures must therefore be able to include in the cyclodextrin cavity. It seems that the length of the alkyl chain between the pyridyl group and the dioxan ring also has an effect on enantioselective complexation. Thus, TA5 and TA6 were poorly resolved. This may be because the site of inclusion is too far removed from the chiral centres in the molecule in these cases for enantioselective interactions to be significant.

The group X also has a noticeable effect on enantioselectivity. Highest selectivity was observed when X = H. Slightly lower selectivity arose when X = OH. Very low selectivity was observed in those cases (TA15, TA16, TA17) where X = OMe. This is indicative perhaps of a steric effect, whereby the inclusion of the aryl ring is hindered by the presence of a comparatively bulky methoxyl group.

The length of the alkyl chain, as given by the value of n, appeared to have very little effect on chromatographic properties. Thus, the selectivities observed for TA11 and TA12 were very similar, with the larger retention of TA12 in both chromatographic systems being attributable to its slightly higher hydrophobicity.

3.6. Other compounds

A number of other racemates were resolved using ß-cyclodextrin mobile phases, as shown in Table 3.7.

No resolution could be achieved for ephedrine, norephedrine, phenylalanine, pheniramine, normetanephrine, mebrophenhydramine, buclizine, ICI1, ICI2, fenoldopam, hydroxyzine, neobenodine, meclizine, telemzepine, tryptophan, metoprolol, prilocaine, AM5(6), bupranolol, bunolol, disopyramide or verapamil. Attempts were also made to separate the enantiomers of orciprenaline, salbutamol, benserazide and 3,4-dihydroxyphenylalanine. These solutes were unretained under all the conditions employed.

Table 3.8 summarises the resolution of other racemates achieved on a Cyclobond I column. In this system, no resolution could be achieved for trihexyphenidyl, bunolol, mebrophenhydramine, bupranolol, oxyphenonium bromide, tetramisole, verapamil, metoprolol, disopyramide, AM5(6), phenindamine, methyl phenidate, tropicamide, and neobenodine. These solutes were retained on the Cyclobond I column, indicating that some inclusion interactions were occurring. Other solutes - pseudo-ephedrine, tryptophan, benserazide, prilocaine, salbutamol and orciprenaline - were unretained on Cyclobond I, using a mobile phase consisting of acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (6:94, v/v). These solutes clearly interact weakly, if at all, with ß-cyclodextrin.

Table 3.7. Resolution of various racemates using beta-cyclodextrin-containing mobile phases

Solute	k'_1	α	R_S	conditions
pseudo-ephedrine	0.29	1.17	0.8	A
brompheniramine	2.9	1.15	1.0	A
carbinoxamine	3.9	1.07	0.8	A
chlorpheniramine	4.8	1.12	1.1	A
benzoin	1.3	1.14	1.0	B
dimethindene	24.4	1.06	0.6	A
chlorthalidone	0.9	1.46	2.6	C
nomifensine	0.9	1.37	1.8	D
methyl phenidate	13.1	1.07	1.4	E
nefopam	3.6	1.11	2.9	E
tetramisole	3.0	1.04	0.7	E
oxyphenonium bromide	6.3	1.07	0.9	E
tropicamide	23.2	1.20	2.9	E
phenindamine	41.0	1.10	1.4	F

Conditions: (A): Column: Zorbax CN (250 x 4.6mm); Mobile phase: acetonitrile - triethylammonium acetate (0.8% TEA, v/v; acetic acid to pH 5.0) (4:96, v/v) containing 18mg/ml beta-cyclodextrin hydrate

(B): Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: dimethyl sulphoxide - water (5:95, v/v) containing 12mg/ml beta-cyclodextrin hydrate

(C): Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: methanol - water (5:95 v/v) containing 12mg/ml beta-cyclodextrin hydrate

Table 3.7 (contd.)

(D); Column: Spherisorb S5CN (250 x 4.6mm); Mobile phase: methanol - aq. triethylammonium acetate (0.85% TEA v/v, acetic acid to pH 4) (5:95, v/v) containing 11mg/ml beta-cyclodextrin hydrate

(E): Column: Zorbax RX C8 (250 x 4.6mm); Mobile phase: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 20mg/ml beta-cyclodextrin hydrate

(F): Column: SGE-100GLC4-C8-8/5 (100 x 4mm); Mobile phase: as D.

Table 3.8 Resolution of racemates on a Cyclobond I (250 x 4.6mm) column.

Solute	k'_1	α	R_S	mobile phase
chlorthalidone	0.6	1.18	1.0	A
benzoin	1.9	1.07	0.7	A
nomifensine	0.2	1.5	1.0	A
nefopam	3.8	1.14	1.2	B

Mobile phases: A: methanol - aq. triethylammonium acetate (0.5% TEA v/v, acetic acid to pH 4) (40:60 v/v)

B: acetonitrile - aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) (12.5:87.5, v/v)

3.7. Determination of ß-cyclodextrin complex formation constants by HPLC

K_f and complex retention values for the enantiomers of chlorthalidone, benzoin, and brompheniramine were determined from the variation of k' with eluent ß-cyclodextrin concentration. The data was fitted to equation 3.11 using MINSQ. The data are illustrated in Figures 3.5, 3.6 and 3.7, and the resulting thermodynamic parameters are shown in Tables 3.9 and 3.10. Good fit of the data to the model was achieved in these cases, as shown by the high values of correlation coefficient obtained.

The complexes of benzoin and chlorthalidone were seen to be unretained under the conditions employed. The high enantioselectivity seen for chlorthalidone was seen to result from a large difference in the K_f values for its enantiomers. Benzoin was more strongly complexed, but there was less difference between the K_f values calculated.

Brompheniramine complexes were seen to be significantly retained under the conditions employed. The K_f values obtained (793 and 750 M^{-1}) were similar to those reported by Mularz (1988) - 741 and 668 M^{-1}.

In similar experiments - methyl mandelate and nomifensine with ß-cyclodextrin - very poor fit of data to equation 3.11 was obtained. This could be due to the breakdown of one or more of the assumptions inherent in equation 3.11 or (more likely) due to failure to adequately control experimental conditions. In particular, variation in stationary phase retentivity during the course of the experiment might be expected to cause difficulties in obtaining meaningful results. It is also important to note that K_f values obtained from these experiments are "apparent" K_f values, and will be different from the true thermodynamic parameters because the experiments were conducted in the presence of acetonitrile and buffer components.

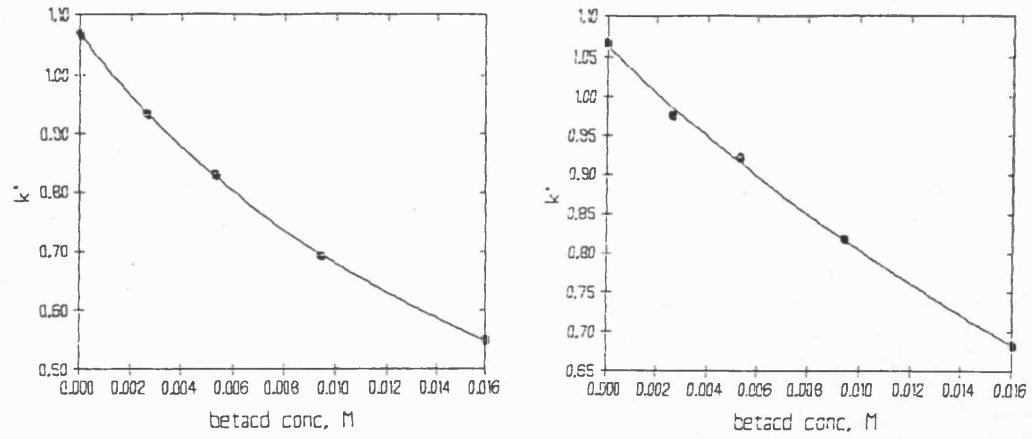


Figure 3.5. Variation of k' with ß-cyclodextrin concentration for chlorthalidone enantiomers. Column: Zorbax CN 250 x 4.6mm; Mobile phase: acetonitrile - water (10:90, v/v) containing ß-cyclodextrin. Data fitted to eqn. 3.11 using MINSQ.

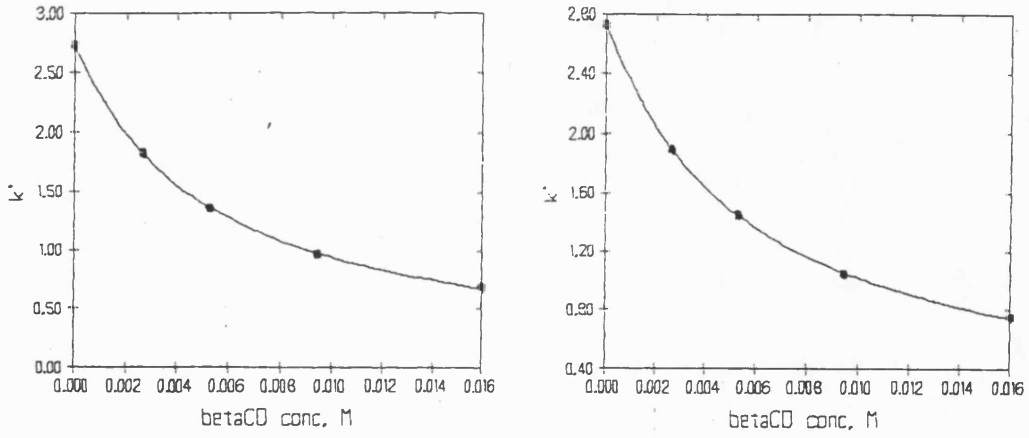


Figure 3.6. Variation of k' with ß-cyclodextrin concetration for benzoin enantiomers. Conditions as figure 3.5.

Table 3.9. Stability and chromatographic properties of the complexes of benzoin and chlorthalidone enantiomers with beta-cyclodextrin on Zorbax CN stationary phase, in acetonitrile - water (10:90, v/v) mobile phases. Data fitted to eqn. 3.11 using MINSQ.

Solute	Parameter	Value	Std. Dev.
R-benzoin	$k'_{complex}$	0.011	0.026
	apparent K_f, M^{-1}	193.0	5.2
	correlation coefficient, r	0.99996	
S-benzoin	$k'_{complex}$	0.020	0.030
	apparent K_f, M^{-1}	171.6	5.3
	correlation coefficient, r	0.99995	
chlorthalidone (peak 1)	$k'_{complex}$	- 0.13	0.04
	apparent K_f, M^{-1}	47.9	2.8
	correlation coefficient, r	0.99994	
chlorthalidone (peak 2)	$k'_{complex}$	- 0.58	0.56
	apparent K_f, M^{-1}	18.7	8.2
	correlation coefficient, r	0.9991	

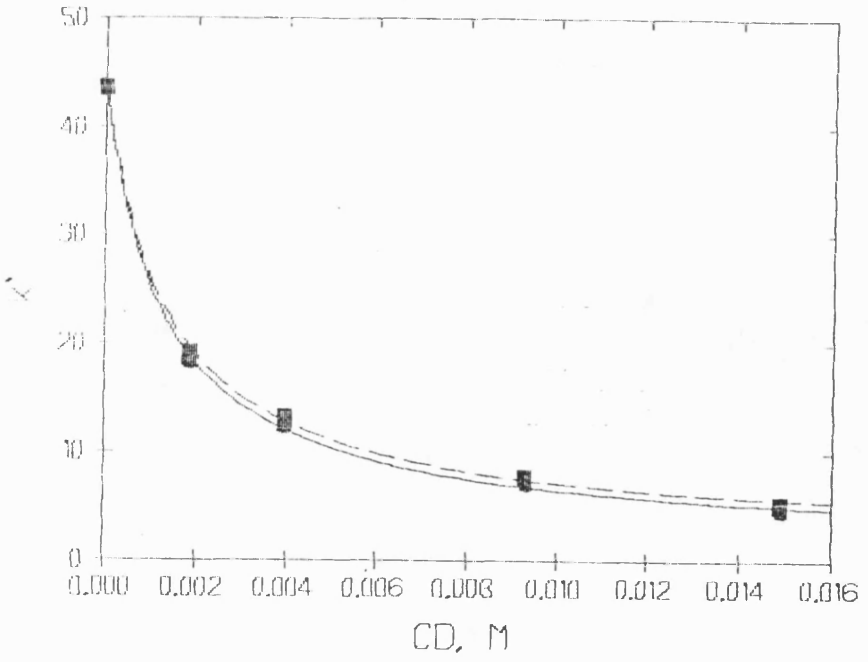


Figure 3.7. Variation of k' with ß-cyclodextrin concentration for brompheniramine enantiomers. Column: SGE-100GL2-C8-30/5. Mobile phase: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90 v/v) containing ß-cyclodextrin. Data fitted to eqn. 3.11 using MINSQ.

Table 3.10. Stability and chromatographic properties of the complexes of brompheniramine enantiomers with beta-cyclodextrin. Column: SGE-100GL2-C8-30/5. Mobile phases: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing beta-cyclodextrin. Data fitted to eqn. 3.11 using MINSQ.

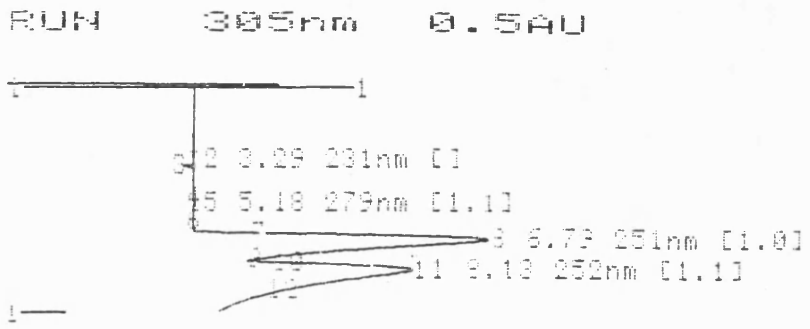
Solute	Parameter	Value	Std. Dev.
brompheniramine (peak 1)	$k'_{complex}$	1.8	0.2
	apparent K_f, M^{-1}	793	23
brompheniramine (peak 2)	$k'_{complex}$	2.3	0.2
	apparent K_f, M^{-1}	750	22
	correlation coefficient, r	0.9997	

3.8. Application of advanced detection techniques to chromatographic studies using cyclodextrin eluents.

3.10.1 Photodiode-array detection

The potential utility of diode-array detection as a confirmatory method for chiral separations is illustrated by Figure 3.8, which shows the output obtained from analysis of the separation of trimeprazine enantiomers.

(a)



(b)

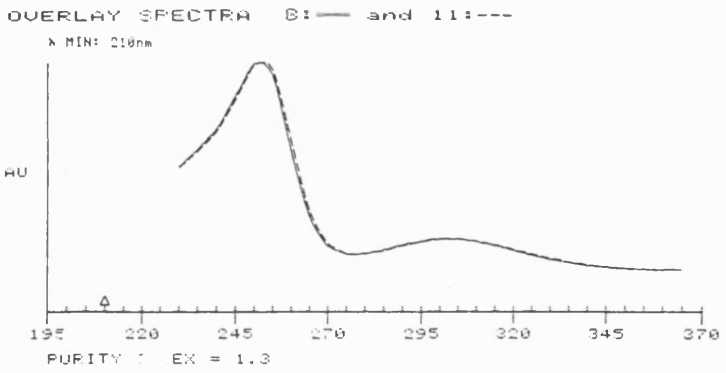


Figure 3.8. Diode-array detector (Perkin-Elmer LC-135) output for separation of trimeprazine enantiomers. (a) real-time chromatogram (b) overlay of apex spectra of peaks for the two enantiomers. Conditions: Column: Zorbax TMS (250 x 4.6mm); Mobile phase: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 5) (4:96, v/v) containing 20mg/ml ß-cyclodextrin hydrate.

Overlaying the spectra obtained at the apex of each peak confirmed that the two peaks arose from species with the same UV absorbance spectrum, thus considerably reducing the possibility of an erroneous "chiral" separation based on the resolution of the desired compound and an impurity. However, since the diastereomeric complexes of two enantiomers with ß-cyclodextrin may have different UV spectra, it is possible that a "false negative" result may be obtained using this approach, which must therefore be used with caution.

3.10.2 Polarimetric detection

The utility of an optical activity detector with ß-cyclodextrin mobile phases was evaluated. As might be expected, the chiral cyclodextrin mobile phase gave rise to high background optical rotation. Whilst the detector "auto-zero" function was able to back-off this background signal to zero, baseline noise and drift was a significant problem and it was rarely possible to reduce the attenuation below the highest level. Sensitivity (never high with such detectors) was therefore considerably reduced compared with what might be achievable using achiral eluents.

A variety of racemates and enantiomers, with varying degrees of resolution under the conditions employed, were chromatographed. For some (*e.g.* promethazine) no signal was detected, even at high column loading (up to 100µg on column.) The best results were achieved for polar solutes such as benzoin and methyl mandelate with relatively high specific rotations. Thus S- benzoin was readily detected at only 6µg on column.

Perhaps the most striking results were obtained for methyl mandelate, where a racemate that showed no resolution by UV detection was clearly seen to be partially resolved using polarimetric detection (as shown in Figure 3.9).

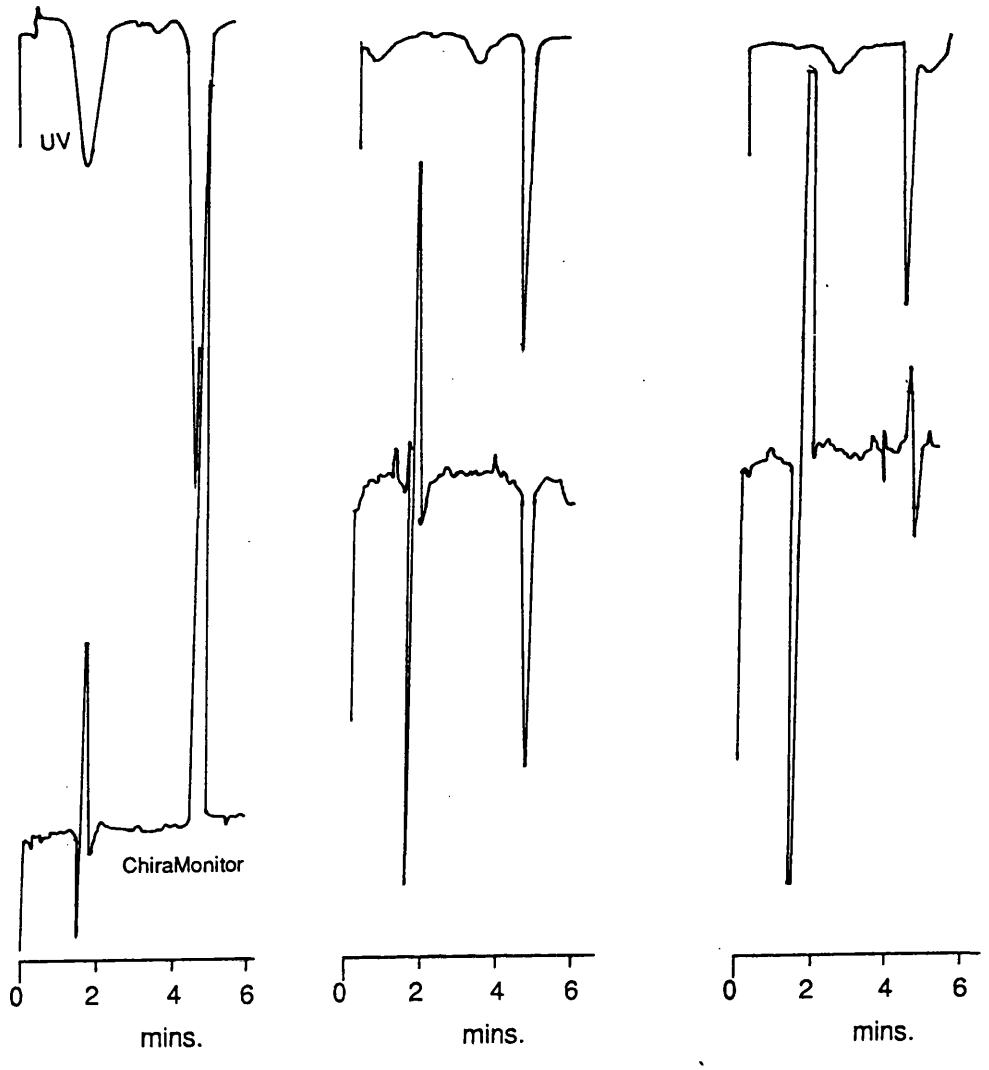


Figure 3.9. Chromatograms obtained using the ACS ChiraMonitor detector (a) S-(+)-methyl mandelate (138µg on col.) (b) R-(-)-methyl mandelate (174µg on col.) (c) RS-methyl mandelate (100µg on col.) UV detector output is shown for comparison.

Conditions: Column: SGE-100GL4-C8-30/5; Mobile phase: acetonitrile - aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) containing 13.5mg/ml ß-cyclodextrin hydrate; ChiraMonitor attenuation: 64; UV detection at 254nm, 2.0 AUFS.

It was noticeable in certain cases that the peaks shown by polarimetric detection for a resolved racemate were by no means of equal and opposite area, as would be the case for a racemate resolved using an achiral mobile phase and a chiral stationary phase. This is clearly seen in the traces obtained for trimeprazine (Figure 3.10).

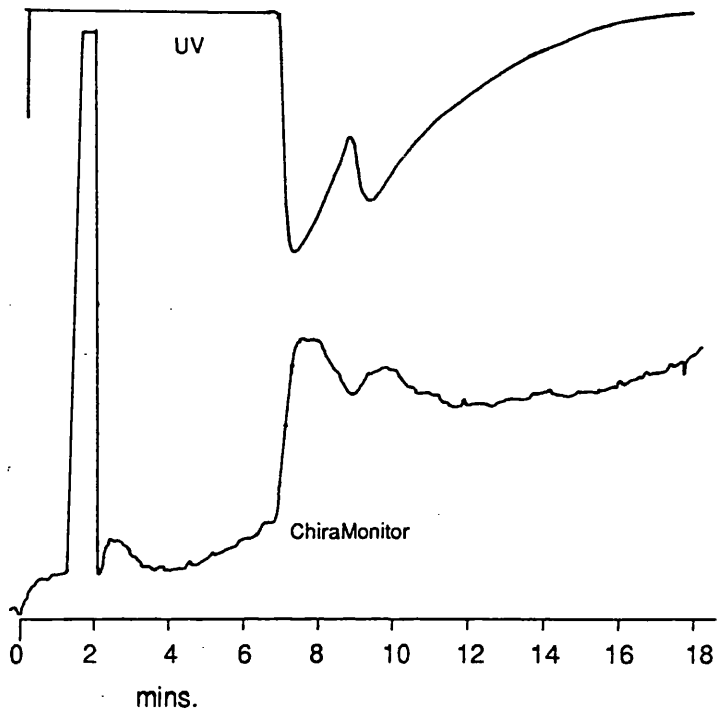


Figure 3.10. Chromatogram obtained using the ACS ChiraMonitor detector, showing resolution of trimeprazine enantiomers (1mg trimeprazine hemi-(+)-tartrate on col.). UV detector output also shown. Conditions as Figure 3.9, except UV detection at 315nm, 2.0AUFS.

This phenomenon may arise due to the fact that the complexes of two enantiomers with ß-cyclodextrin in the mobile phase are diastereomeric, and therefore may not have equal and opposite optical rotations. This factor may limit the quantitative application of polarimetric detection when using chiral mobile phases. It thus seems that the main application of this detection method will be in semi-preparative separations, where sensitivity is not limiting, and in qualitative confirmation of chiral separations.

3.9 Use of derivatised cyclodextrins as mobile phase additives

A number of experiments were conducted in which derivatised ß-cyclodextrins (dimethyl, and various hydroxyalkyl derivatives) were used as mobile phase additives. In contrast to previously reported studies (Zukowski *et al* (1988), Clark and Mama (1989a)), stationary phases of moderate hydrophobicity and mobile phases with low organic modifier contents were employed, in order to compare more directly the influence of these additives with ß-cyclodextrin itself.

3.9.1. Comparison of selectivity and retention obtained under identical conditions using various additives.

The retention, selectivity and resolution observed for a number of racemic solutes on an SGE-100GL2-C8-30/5 column with mobile phases consisting of acetonitrile-aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90 v/v) containing 7.2 mmol l^{-1} of ß-cyclodextrin, methyl-ß-cyclodextrin (DS=1.8), hydroxyethyl-ß-cyclodextrin (MS=1.0), or hydroxypropyl-ß-cyclodextrin (MS=0.9) are shown in Table 3.11

In all cases, resolution of enantiomers was much poorer using the derivatised cyclodextrins, due to lower selectivity. The hydroxyethyl derivative gave highest resolution of the three derivatives. No resolution at all was obtained with the methylated cyclodextrin.

The retention times of the solutes investigated were shorter with the methyl-ß-cyclodextrin-containing eluent than with the equivalent mobile phase containing ß-cyclodextrin itself. This indicates that the methyl-ß-cyclodextrin is more effective at eluting solutes than the parent compound, which is most likely to be due to stronger complex formation in the mobile phase. However, it is clear that this stronger complexation is not accompanied by an increase in enantioselectivity. Thus, the modes of inclusion of the solutes in the two cyclodextrin cavities must be different.

Table 3.11. Resolution of enantiomers using mobile phases containing beta-cyclodextrin and derivatives. Conditions as text.

solute	betaCD			OH-Et-betaCD		
	k'_1	α	R_S	k'_1	α	R_S
trimeprazine	9.8	1.2	1.7	10.8	1.18	1.3
brompheniramine	7.0	1.10	0.75	9.0	1.06	0.6
chlorpheniramine	10.0	1.05	0.6	9.5	not resolved	
benzoin	10.8	1.08	1.0	14.9	1.05	0.8
chlorthalidone				6.1	1.10	1.1

solute	OH-Pr-betaCD			Me-betaCD	
	k'_1	α	R_s	k'_1	
trimeprazine	10.2	1.14	0.75	5.5	not resolved
brompheniramine	7.9	not resolved		6.3	not resolved
chlorpheniramine	7.6	not resolved		5.5	not resolved
benzoin	10.3	1.05	<0.5	7.1	not resolved
chlorthalidone	3.5	1.09	0.6	4.0	not resolved

3.9.2. Effect of degree of substitution on performance of derivatised cyclodextrins.

A hydroxyethyl- substituted ß-cyclodextrin with a lower degree of average molar substitution (MS = 0.6) was found to give improved resolution of all the racemates investigated, as shown in Table 3.12. In some cases, higher selectivity than that given by underivatised ß-cyclodextrin under similar conditions was observed. In these cases, the lower degree of substitution may be providing optimum number of hydrogen-bonding sites and steric bulk around the cavity rim for enantioselectivity.

3.9.3. Use of high additive levels

Tables 3.12, 3.13 and 3.14 summarise data obtained using much higher additive concentrations than would be possible with underivatised ß-cyclodextrin, on a more hydrophobic column than used previously (Spherisorb C6).

Table 3.12. Resolution of enantiomers using hydroxyethyl-beta-cyclodextrin (MS = 0.6). Column: Spherisorb C6 (150 x 3mm); Mobile phase: acetonitrile: aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) (10:90 v/v)

Solute	20mg/ml CD in eluent			100mg/ml CD in eluent		
	k'_1	α	R_S	k'_1	α	R_S
nomifensine	7.3	1.39	2.1	1.8	1.35	1.4
chlorthaiidone	16.8	1.17	1.5	6.4	1.33	1.4
brompheniramine	18.3	1.08	1.1	5.9	1.13	0.8
benzoin	29.9	1.09	1.3	8.4	1.13	1.1
chlorpheniramine	20.2	1.06	1.0	6.5	1.10	0.8
trimeprazine	29.1	1.17	1.5	4.9	1.18	1.3

Table 3.13. Resolution of enantiomers using hydroxypropyl-beta-cyclodextrin (MS = 0.9) on Spherisorb C6. Conditions as Table 3.13

Solute	20mg/ml CD in eluent			100mg/ml CD in eluent		
	k'_1	α	R_S	k'_1	α	R_S
nomifensine	7.4	1.15	1.5	2.1	1.19	1.1
chlorthalidone	13.7	1.10	1.5	7.1	1.28	1.3
brompheniramine	16.0	1.06	0.9	4.5	1.06	
trimeprazine	25.6	1.18	1.8			

Table 3.14. Resolution of enantiomers using methyl-beta-cyclodextrin (DS = 1.8) on Spherisorb C6. Conditions as Table 3.13

solute	100mg/ml CD in eluent		
	k'_1	α	R_S
nomifensine	1.9	1.26	1.1
chlorthalidone	4.2	not resolved	
brompheniramine	5.3	not resolved	

With hydroxyethyl-ß-cyclodextrin (DS = 0.6), some increases in selectivity were obtained using high additive levels, but resolution was in general lower owing to sub-optimal retention and a marked drop in column efficiency. This deterioration in column performance probably reflects the increased viscosity of eluents containing high additive levels, as discussed by Armstrong *et al* (1988b), resulting in poorer mobile phase mass transfer.

With the other derivatives, improved resolution was obtained at high additive levels. However, this was still lower than obtained using the underivatised cyclodextrin. Comparison of the results obtained for hydroxypropyl-ß-cyclodextrin (MS = 0.9) at the two additive levels employed leads to similar conclusions to those above. In this case, even at 100mg/ml in the eluent, selectivity was lower than has been observed using much lower concentrations of underivatised ß-cyclodextrin. This suggests that the inherent enantioselectivity of the inclusion process has been lessened by derivatisation. For methyl-ß-cyclodextrin, some resolution was observed at high additive levels where none had been using lower levels of this additive, but selectivity was again low compared to that achievable using underivatised ß-cyclodextrin.

Chapter 4

Results: Semi-preparative resolution of enantiomers using ß-cyclodextrin-containing mobile phases

4.1 Introduction

Cyclodextrins have long been applied to the preparative resolution of complexed solutes, including enantiomers. Techniques involved include precipitation, differential reactivity, gel chromatography and, most recently, HPLC.

4.1.1. Resolution of enantiomers by stereoselective precipitation using cyclodextrins.

The diastereomeric complexes formed between a cyclodextrin and a racemic substrate may differ in their solubilities, as well as their formation constants. Therefore, if cyclodextrin is added to increase the solubility of a poorly soluble racemic mixture, the resulting solution may be enriched in one enantiomer. This was first exploited to effect chiral resolution by Cramer and Dietsche (1959). They used ß-cyclodextrin to resolve carboxylic acid esters by this approach, and achieved optical purities of 3 to 10% after one separation step, which could be increased by repeated precipitation or recrystallisation steps. Other racemates, including phosphinates (Benschop and Van den Berg (1970)) and sulphinates (Mikolajczyk and Drabowitz (1978)) have subsequently been resolved by this method, with one-step optical purities of up to 80% achieved in some cases.

The chief drawback of this approach is that, like the classical resolution method of fractional crystallisation of diastereomeric derivatives, many steps are usually required to achieve high optical purities. However, recovery of the resolved enantiomers from their cyclodextrin complexes is somewhat easier than the reversal of derivatisation required in the classical approach.

4.1.2 Enantioselective catalysis by cyclodextrins.

Cyclodextrins are known to catalyse effectively certain reactions, particularly hydrolyses and trans-acylations (Hinze (1981)). Such catalysis may be enantioselective, *i.e.* the cyclodextrin

may catalyse the reaction of one enantiomer of a racemate faster than the other, leading to partial resolution of the racemic product. This principle has been applied effectively to the resolution of racemic organophosphates and other substrates. In the best case, ±-isopropyl-2-dimethylaminoethylmethylphosphonthioate, a rate differential of 110 in alpha-cyclodextrin-catalysed base hydrolysis was reported (Van Hooidonk (1972)). In a recent report (Coates *et al* (1991)), a potentially effective method for the resolution of ibuprofen enantiomers has been outlined. This involves enantioselective hydrolysis of a cyclodextrin-ibuprofen ester, with the diastereomer derived from (R)-ibuprofen being more than 10 times more susceptible to hydrolysis than that derived from (S)-ibuprofen.

The application of this technique has proved to be limited to certain substrates. A major drawback is that chemical change in the substrate may be inherent in the method.

4.1.3 Preparative separations on cyclodextrin polymer gels.

Cyclodextrin polymer gels, produced by cross-linking with epichlorohydrin, have been used for preparative chromatographic separations, both achiral and chiral. Zsadon *et al* (1983) resolved the enantiomers of a number of indole alkaloids by this approach, using a 50x900mm column packed with ß-cyclodextrin polymer. ±-Vincadifformine was resolved at optical purities of 98.3% and 92.5% for the two enantiomers in 92.5% yield at a rate of 17mg/hour. Increasing throughput to 25 mg racemate per hour reduced optical purity to 87.5% and 81.6% for the two enantiomers.

The chief drawback of this technique, when compared to preparative HPLC, is the large particle size of such cyclodextrin gels, resulting in poor mass transfer and hence low efficiency. Other gels, where the cyclodextrin is immobilised on the surface of a non-complexing polymer such as polyacrylamide (Tanaka *et al* (1981)), exhibit higher efficiency but much lower capacity, and so have not been applied to preparative separations.

4.1.4 Semi-preparative HPLC on cyclodextrin silicas

Cyclodextrin-silica HPLC phases generally have lower loading capacities than conventional reversed-phase materials (Vigh *et al* (1989)). Consequently, reports of preparative HPLC separations in the conventional (elution) mode are few and far between. Florance *et al* (1987) reported the attempted semi-preparative resolution of enantiomers of a cyclic peptide at hundred milligram scale on a 1 inch i.d. Cyclobond I column. Resolution was reported to be poor, however.

In a recent series of publications, Vigh *et al* (1989, 1989a, 1990) have described the effective application of Cyclobond I columns to preparative separations in displacement mode.

Displacement chromatography has recently been re-discovered as a potentially useful technique for achieving large-scale separations. In reversed-phase elution chromatography, which is the mode most applicable to small-scale analytical separations, solutes are partitioned between mobile and stationary phases and are eluted due to the presence of organic modifiers in the mobile phase. In the past, overloaded elution chromtatography has been much used for preparative separations, owing to its close relationship to analytical HPLC, allowing facile method development. In displacement chromatography, solutes are introduced to the column in a mobile phase which does not effect their rapid elution. A displacer solution, containing a high concentration of a substance which has a stronger affinity for the stationary phase than the solutes, is then pumped through the column. This displaces the solutes from the stationary phase in order of their adsorption strengths. If these are sufficiently different, and the column is efficient, then solutes will be fully resolved and will emerge from the column in turn. Horvath (1985) has argued that throughput can be increased by an order of magnitude in displacement mode compared to overloaded elution mode.

Vigh has used displacement HPLC on Cyclobond I to achieve impressive preparative separations of positional, geometric and optical isomers, using normal or reversed-phase solvents (Vigh *et al* (1989, 1989a, 1990). Using two 250x4.6mm Cyclobond I columns in series, up to 6mg of various racemates (mephobarbital, hexobarbital, dansylleucine, dansylvaline) were resolved in high yield and optical purity in run times of 3-6 hours. In all these cases, the elution mode selectivities at low loading were less than 1.1 under the conditions used, indicating that preparative separations in elution mode would not have been realistic.

4.1.5 Preparative chromatographic separations using cyclodextrin-containing eluents

As with all chiral separation methods using mobile phase additives, application of cyclodextrin eluents to preparative separations has been limited by the problem of recovering the resolved enantiomers free from the chiral additive (Pirkle and Sikkenga (1976)). Sato and Suzuki (1985) separated the enantiomers of warfarin and mandelic acid as their ß-cyclodextrin complexes on Sephadex gel. Optical purities of around 30% were achieved, with throughputs of up to 2mg per hour on a 400 x 25mm column. The enantiomers were recovered by freeze-drying followed by addition of acetone to break up the complexes and precipitate out the cyclodextrin.

The most significant report in this area has come from Harada *et al* (1989), who used alpha-cyclodextrin as a mobile phase additive to achieve the medium-pressure liquid chromatographic resolution of the enantiomers of some chiral ferrocenes on a polyamide gel stationary phase. High selectivities (αup to 1.64) were obtained and throughputs of up to 20mg racemate per hour were achieved on a 500 x 8mm column. The resolved enantiomers were recovered by solvent extraction (although no experimental detail on this point was given), and it was reported that the alpha-cyclodextrin could also be recovered and re-used (which was economically advantageous).

Only brief mention of the use of cyclodextrins as eluent additives in preparative RP-HPLC has appeared in the literature. Thus, Sybilska *et al* (1986) have reported the use of ß-cyclodextrin in the mobile phase to achieve the preparative separation of enantiomers of mephenytoin and hexobarbital on a 250 x 8mm Lichroprep RP18 column. However, no data for these separations were given , and there was no indication that attempts had been made to recover the enantiomers from the mobile phase after resolution. It is clear that the good resolutions obtained in analytical-scale HPLC using cyclodextrins have yet to be exploited to their full potential on a larger scale.

4.1.6 Enantiomer separations using cyclodextrin-containing liquid membranes.

The potential utility of liquid membranes containing chiral additives to achieve very large scale enantiomer separations has recently been demonstrated (Pirkle, W.H., reported at *2nd. Int. Symp. Chiral Sepns,* Guildford (1989)). The principle of this technique is that the the chiral additive acts to solubilise the solutes in a liquid membrane separating two portions of a solvent with which the liquid is immiscible. Solutes are thereby transported across the membrane. The selectivity induced by the chiral additive may result in enrichment of one enantiomer from a racemate. Preparative resolutions at high optical purity may therefore be achieved by the use of a number of such liquid membrane systems in series.

Armstrong and Jin (1987c) have described the application of this approach using cyclodextrins to selectively transport solutes across an aqueous liquid membrane separating two portions of ether. By this means, the potential for preparative resolution of a number of racemates (derivatives of ferrocene and nicotine, a crown ether, mephenytoin and disopyramide) was demonstrated. The initial rates of transport of two enantiomers across the membrane were found to differ by factors of up to 17.

This technique has enormous potential for process-scale separations, since it can be

operated on a continuous-flow basis and the chiral additive is not consumed. However, no further reports of its use with cyclodextrins have appeared since Armstrong's publication in 1987.

4.2 Trimeprazine

4.2.1 Effect of trimeprazine loading on resolution.

The variation in resolution of trimeprazine enantiomers on a wide-pore C8 column (100x4mm) with concentration of injected solution (at constant injection volume) using a ß-cyclodextrin-containing eluent is illustrated in Figure 4.1, and quantified in Table 4.1.

Table 4.1. Effect of trimeprazine loading on resolution. Conditions as Figure 4.1. Recovery and optical purities of collected fractions (assayed by reinjection into the system) are also shown.

Column loading	resolution		Peak 1		Peak 2	
mg	R_s	%CRF.	recovery %	e.e., %	recovery %	e.e, %
0.018	1.9	99				
0.044	1.4					
0.088	1.2					
0.156	1.0	94				
0.195	0.8					
0.389	0.8	61				
0.778	0.5		81		100	
1.98	0.6	36	93	91	100	88

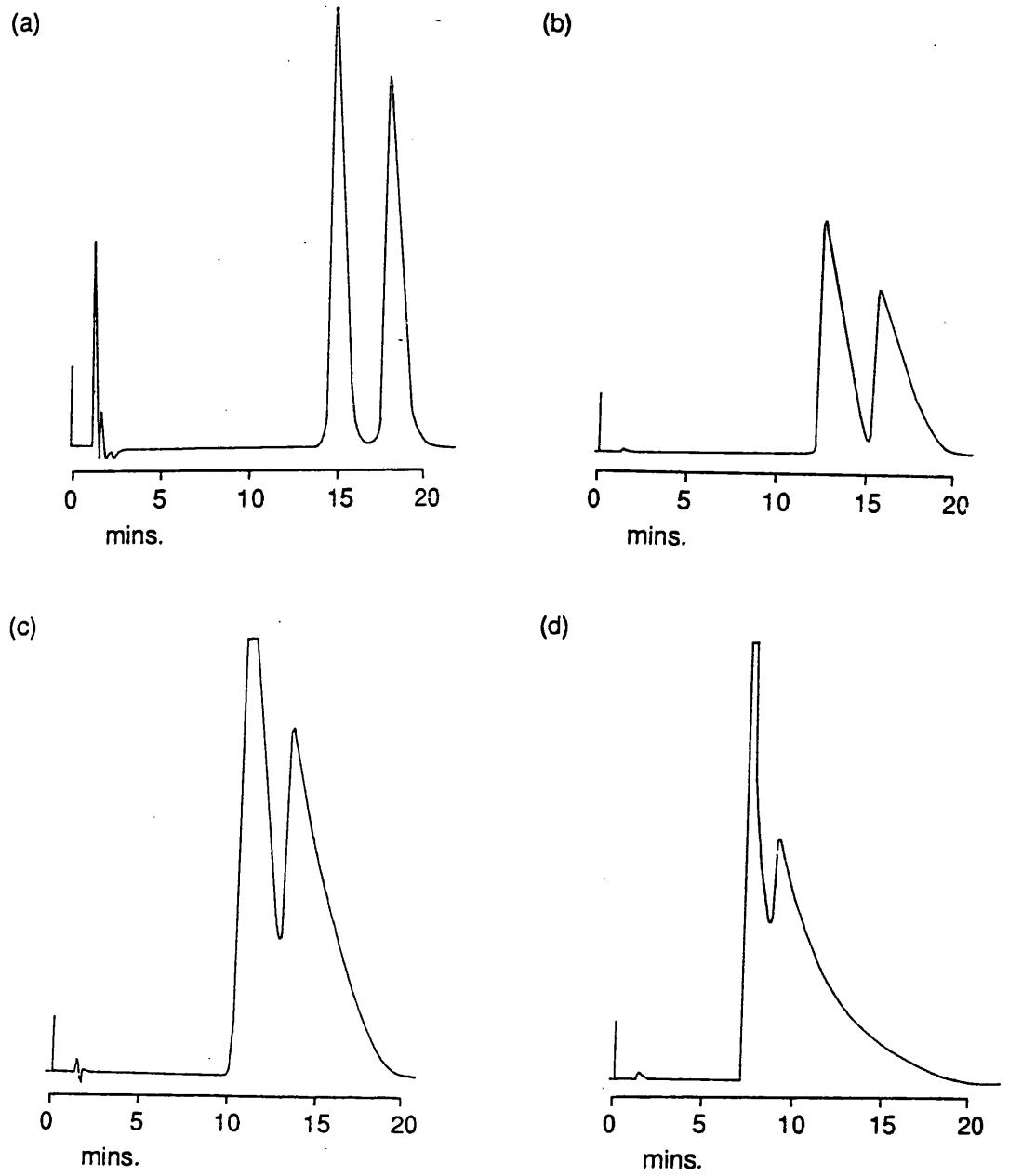


Figure 4.1. Resolution of trimeprazine enantiomers on SGE-100GLC4-C8-30/5 column (containing 0.77g packing material). Mobile phase: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 13.5mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75ml/min. Temperature: 20°C. Injection volume: 100µl. Sample solution.: ±-Trimeprazine hemi-(+)-tartrate in water. Column loading: (a) 0.018mg (b) 0.16mg (c) 0.4mg (d) 1.98mg. UV detection: (a) 300nm 0.2AUFS (b) and (c) 300nm 2.0AUFS (d) 340nm 2.0AUFS.

Figure 4.1 shows that the trimeprazine enantiomers were baseline resolved at low column loadings. As the loading was increased, retention fell and resolution was reduced. Such behaviour is consistent with that generally reported in overloaded elution chromatography (McDonald and Bidlingmeyer (1987)). This indicates that saturation of stationary phase sites is the primary factor operative on overloading, rather than saturation of the complexing ability of the cyclodextrin in the mobile phase, which might be expected to lead to increases in retention on column overloading. Some resolution was observed at very high column loadings, up to 2mg on this small column.

Table 4.1 also shows the recovery and optical purity of collected fractions, determined by re-injection into the system and comparison of peak areas of the two enantiomers. The high optical purities obtained indicated the possibility of achieving mg-scale separations of trimeprazine enantiomers using this system.

The effects of injection volume and sample concentration on resolution were investigated. The results obtained are illustrated in Figure 4.2.

Peak shapes were significantly poorer on injecting a 40mg/ml sample concentration. Little difference was observed between injections of 10 or 20mg/ml samples (at constant 1mg loading).

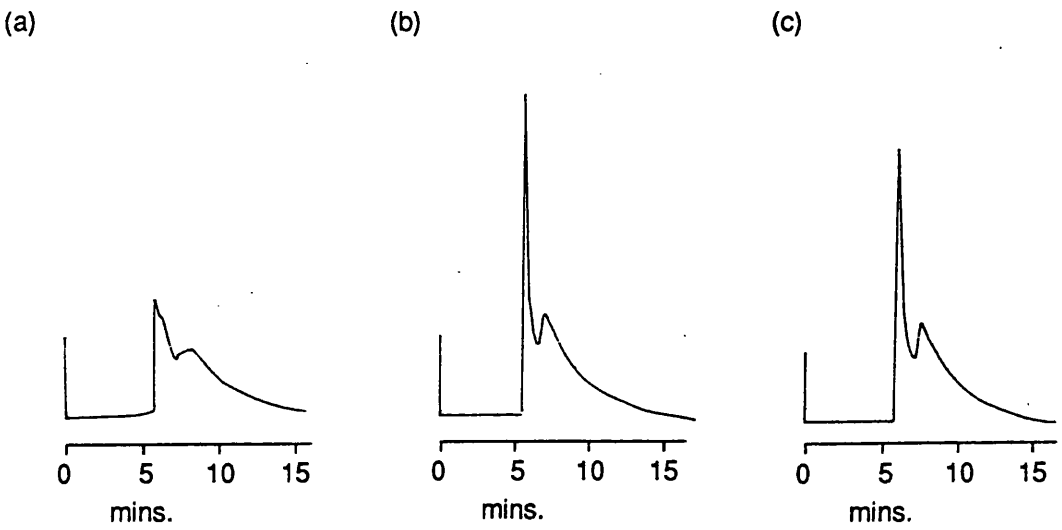


Figure 4.2. Variation in resolution of trimeprazine enantiomers with injection volume and sample concentration. Column: SGE-100GLC4-C8-30/5. Mobile phase: acetonitrile - triethylammonium acetate buffer (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 9mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75mg/ml. Sample solution: ±-Trimeprazine hemi-(+)-tartrate in mobile phase. Injection conditions: (a) 25µl x 40mg/ml (b) 50µl x 20mg/ml (c) 100µl x 10mg/ml.

4.2.2 Effect of mobile phase composition and stationary phase type on resolution of trimeprazine enantiomers.

The effect of varying the acetonitrile content of the eluent on resolution of trimeprazine enantiomers at low column loadings was investigated. The results obtained are shown in Table 4.2.

In the absence of cyclodextrin, an increase in eluent organic modifier content in RP-HPLC causes a decrease in retention. In this case the opposite trend was observed. This may be attributed to the competitive inclusion of the organic modifier reducing the eluting ability of the cyclodextrin in the mobile phase as the organic content was increased.

Table 4.2. Effect of eluent acetonitrile content on resolution of trimeprazine enantiomers. Column: SGE-100GLC4-C8-30/5. Mobile phase: ACN - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) containing 13.5mg/ml beta-cyclodextrin hydrate. Flow rate: 0.75ml/min. Solute: Trimeprazine tartrate (2µg on column)

[ACN] in eluent, % v/v	k'_1	α	R_s	$N_{w1/2}$ m^{-1} (peak 2)
0	5.8	1.30	1.29	5943
5	6.1	1.28	1.91	15283
10	7.0	1.24	2.35	32355

While selectivity was reduced as acetonitrile content was increased, resolution was improved due to a substantial increase in column efficiency (as measured by the number of theoretical plates, N). This is attributable to the "wetting" of the hydrophobic stationary phase by the organic modifier, improving mass transfer.

The effect of ß-cyclodextrin content of the mobile phase on resolution is illustrated in Table 4.3 (at low column loading) and Figure 4.3 (at high column loading).

Table 4.3. Effect of beta-cyclodextrin hydrate concentration in eluent on resolution of trimeprazine enantiomers. Conditions as Figure 4.3, except column loading: 2µg.

eluent [BetaCD], mg/ml	k'_1	α	R_s	$N_{w1/2}$ m^{-1} (peak 2)
6.8	12.4	1.23	2.06	24533
9.0	9.3	1.24	2.10	22945
13.5	7.0	1.24	2.35	32355

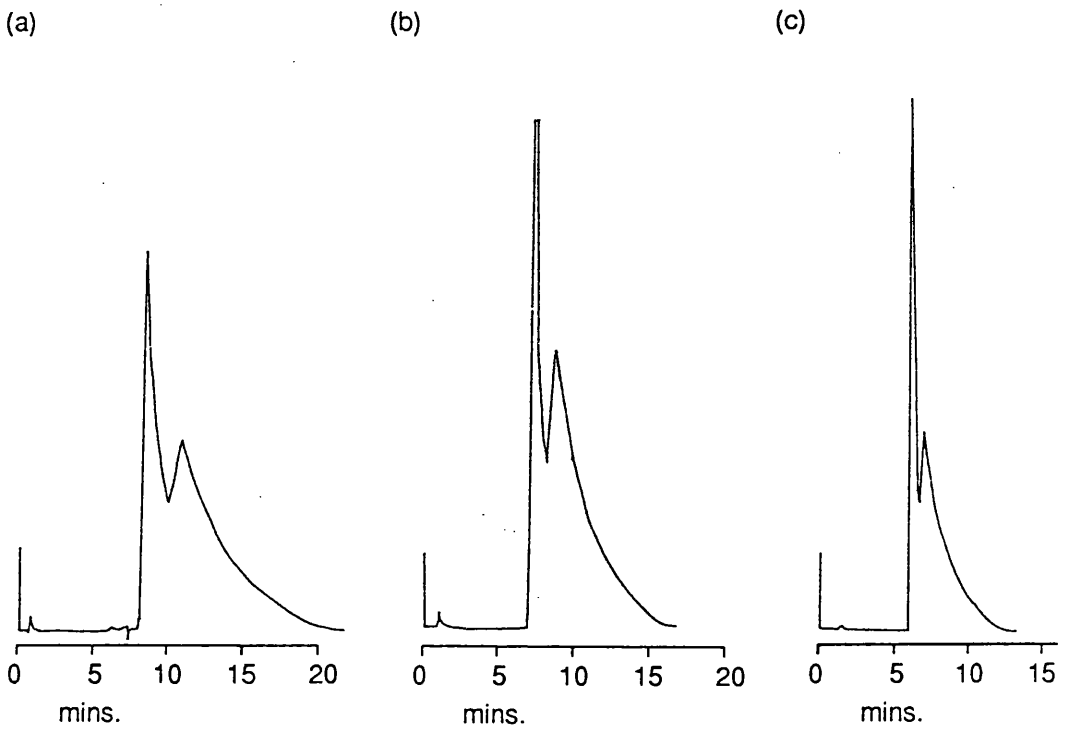


Figure 4.3. Effect of ß-cyclodextrin concentration in eluent on resolution of trimeprazine enantiomers on SGE-100GLC4-C8-30/5 column. Mobile phase: acetonitrile - aq. triethylammonium acetate buffer (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing (a) 6.75mg/ml (b) 9.0mg/ml (c) 13.5mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75ml/min. Loading: 1mg ±-trimeprazine hemi-(+)-tartrate on column.

Selectivity and resolution were increased, and retention time reduced, on increasing the cyclodextrin concentration, due to increasing degree of complexation. However, 9mg/ml was deemed to be the most appropriate concentration for semi-preparative separations using this system, since the slightly increased run time would allow greater margin for error in fraction collection times.

Triethylamine was incorporated in all the eluents employed. It was found to significantly reduce peak tailing, and improve resolution. An eluent concentration of 0.8% v/v was found to be optimal. There was little improvement in resolution on increasing the triethylamine content above this level.

The separation of trimeprazine enantiomers on a column with higher carbon loading was investigated. This gave much longer retention times without improvement in resolution, as shown in Figure 4.4.

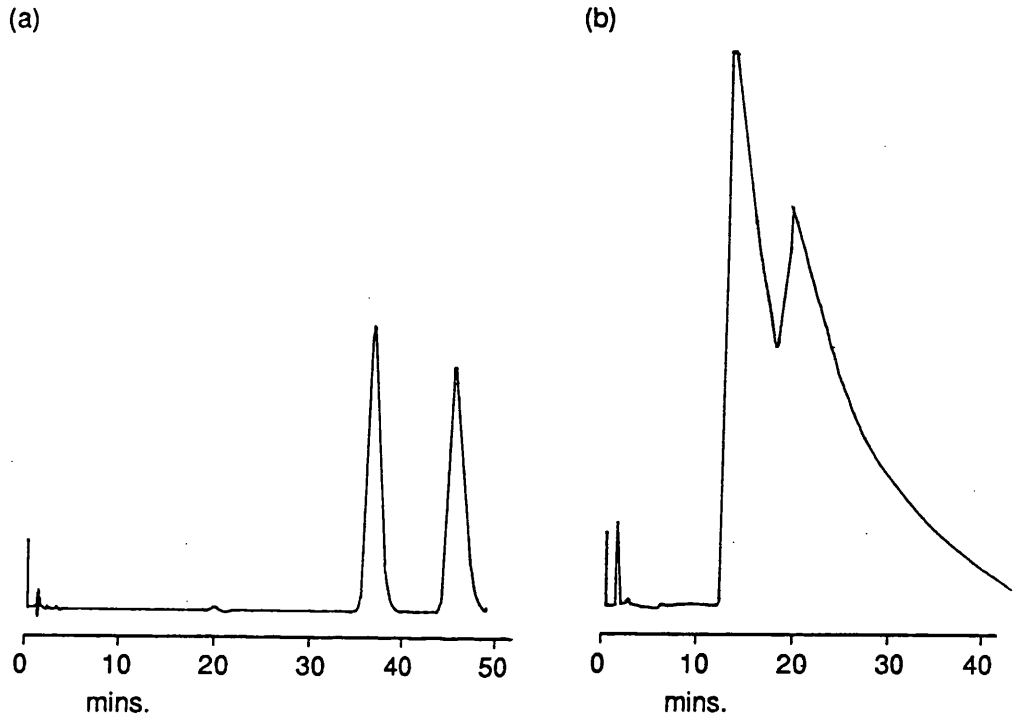


Figure 4.4 Resolution of trimeprazine enantiomers on a SGE-100GL4-C8-8/5 column. Mobile phase: acetonitrile - aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) (10:90 v/v) containing 13.5mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75ml/min. Loading: (a) 5µg; (b) 2mg ±-trimeprazine heml-(+)-tartrate on column.

It was clear that throughput would be reduced under these conditions. Retention time could have been reduced by using a higher acetonitrile content in the mobile phase, but this would have resulted in lower selectivity. The wide-pore C8 material, with low carbon loading, was therefore the most appropriate choice for this separation. The differences in retentivity of the two materials made it impossible to assess whether the differing pore sizes of the two stationary phases had any effect.

The use of a "base-deactivated" reversed-phase stationary phase was investigated as a means of reducing peak tailing without the use of amine modifiers. As shown in Figure 4.5a, resolution on this column was quite poor, and its retentivity rather too high (necessitating the use of higher organic content in the mobile phase than optimal).

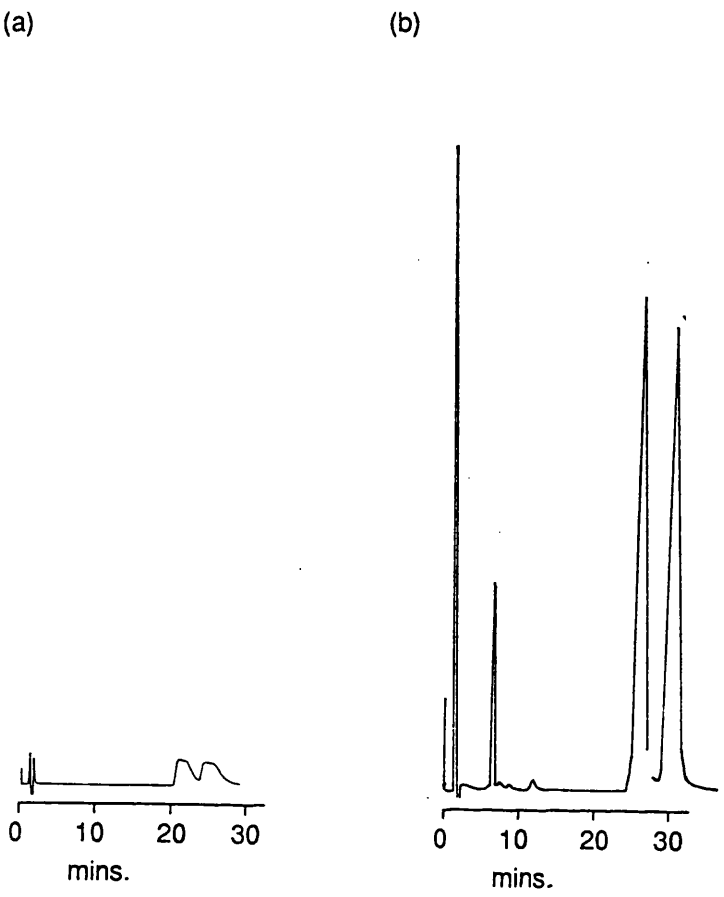


Figure 4.5. Resolution of trimeprazine enantiomers on Hypersil BDS (150x2.1mm). Column loading: 2µg ±-trimeprazine tartrate. (a) Mobile phase: acetonitrile - aq. NaH_2PO_4 (0.05M, H_3PO_4 to pH 4) (20:80, v/v) containing 25mg/ml ß-cyclodextrin hydrate. (b) Mobile phase: acetonitrile - aq. triethylammonium acetate (0.4% TEA v/v, acetic acid to pH 4) (20:80, v/v) containing 20mg/ml ß-cyclodextrin hydrate.

Figure 4.5b shows that an improvement in resolution was obtained by the use of a triethylamine-containing mobile phase on this column, indicating that silanol effects are still operative on this material (i.e. that the base-deactivation is far from complete). Retention was still high, and so little advantage was seen in the use of this phase.

Sodium hexanesulphonate was investigated as a possible alternative to the use of triethylamine-containing mobile phases. The ion-pairing agent increased retention, necessitating the use of an increased acetonitrile content in the eluent. This factor, coupled with poor peak shapes in the absence of triethylamine, led to poor resolution. The ion-pairing agent had no apparent effect on selectivity. This was probably because, in an eluent containing only 20% v/v acetonitrile, adsorption of ion-pairing agent onto the stationary phase (*i.e.* "dynamic ion-exchange") was probably the predominant retention mechanism rather than ion-pair formation in the mobile phase.

4.2.3 Effect of temperature on capacity of Lichrosorb RP18 stationary phase for trimeprazine

The potential utility of Lichrosorb RP18, a very hydrophobic stationary phase (carbon loading 17% w/w) for the recovery of trimeprazine enantiomers following chiral separation using a ß-cyclodextrin-containing eluent was investigated in a series of "breakthough" experiments. The effect of temperature on capacity was also investigated, with a view to disrupting complexation of the trimeprazine by the cyclodextrin at high temperature, thereby perhaps increasing retentivity. The results obtained are illustrated in Figure 4.6.

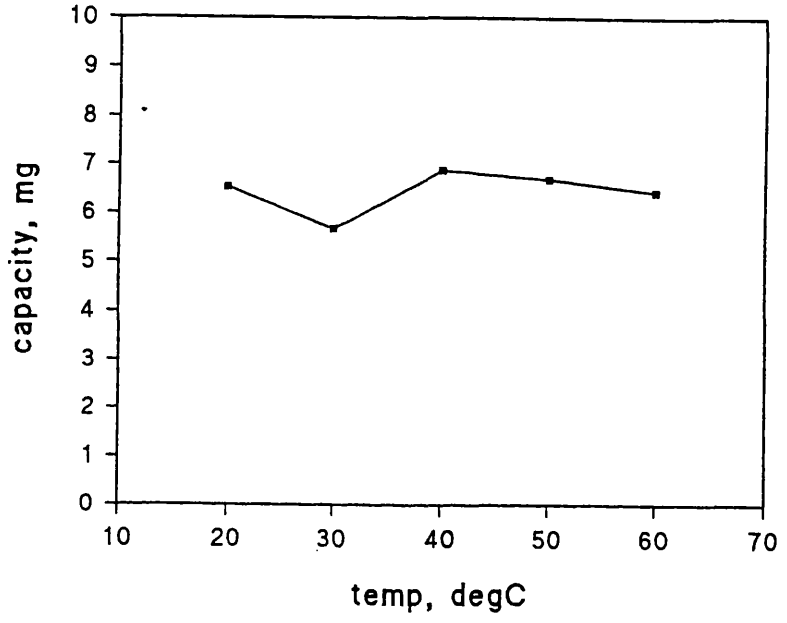


Figure 4.6. Variation in capacity of Lichrospher RP18 for ±-trimeprazine with temperature. Column dimensions: 100x4.6mm. (containing 1.0g packing material). Loading solution: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 13.5mg/ml ß-cyclodextrin hydrate and 0.1mg/ml ±-trimeprazine hemi-(+)-tartrate. Flow rate: 1ml/min.

At all temperatures across the range studied, the capacity of the column was found to be

about 6mg per gram of packing material, which indicated that this phase was likely to be suitable for the intended purpose. Significant variation in capacity with temperature was not observed, indicating that any effects due to the disruption of cyclodextrin concentration at high temperature were cancelled out by the change in inherent capacity of the stationary phase.

4.2.4 Application of the Promis 2 autosampler with integrated stream switching (ISS) facility to the on-line recovery of trimeprazine enantiomers following chromatographic separation.

The ISS facility of the LDC Promis 2 autosampler, consisting of two Rheodyne 7000 six-port switching valves with pneumatic actuators controlled by the autosampler, allowed the possibility of on-line recovery of trimeprazine enantiomers following their separation. Two Lichroprep RP18 columns were used to recover the enantiomers free from ß-cyclodextrin. The high capacity of this phase allowed the resolution of 12-14 1mg injections of racemate on the separating column, before the recovery columns were flushed free of cyclodextrin and the resolved enantiomers eluted.

Several semi-preparative runs were carried out, using differing procedures for flushing the recovery columns free of mobile phase components and eluting the trimeprazine from the recovery columns, as outlined in Table 4.4 The optical purities of the recovered trimeprazine fractions were assayed by re-injection into the system. Typical calibration data from this assay is shown in Table 4.5.

The results of the semi-prep runs listed in Table 4.4 are summarised in Table 4.6. The capacity of recovery column 2 was invariably reached first, presumably because of the greater width and hence longer fraction collection time of peak 2. Consequently, losses of trimeprazine from recovery column 2 on flushing off the cyclodextrin were greater, and recoveries of peak 2 generally lower.

Table 4.4. Summary of semi-preparative procedures employed.

Run	eluent-[BetaCD], mg/ml	flush procedure	elution procedure	loading, mg/enantiomer
1	13.5	1	1	6.0
2	13.5	2	2	7.0
3	13.5	2	2	6.5
4	13.5	2	3	6.0
5	13.5	3	4	6.0
6	9.0	4	4	8.0

Flush procedures: 1. 20ml x acetonitrile - water (20:80, v/v)

2. 5ml x acetonitrile - water (20:80, v/v)
3. 5ml x tetrahydrofuran - water (20:80, v/v)
4. 2.5ml x water + 5ml x acetonitrile - water (10:90, v/v)

Elution procedures: 1. 20ml x acetonitrile - TFA - water (50:0.1:49.9, v/v)

2. 30ml x acetonitrile - TFA - water (50:0.1:49.9, v/v)
3. 25ml x acetonitrile - TFA - water (50:0.1:49.9, v/v)
4. 25ml x acetonitrile - TFA - water (70:0.1:29.9, v/v)

Table 4.5. Calibration data for chiral trimeprazine assay.

Conditions: Column: SGE-100GLC4-C8-30/5. Mobile phase: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 9mg/ml beta-cyclodextrin hydrate. Internal standard: levomepromazine (50µg/ml). Detection: UV 300nm. Integration: Spectra-Physics SP4270.

[trimep. tart.],	peak heights			peak height ratios	
µg/ml/enantiomer	trimeprazine		int.std.		
	peak 1	peak 2		peak 1	peak 2
2.61	4124	4009	135149	0.0305	0.0296
	4717	4361	126593	0.0373	0.0344
26.1	42722	39765	135646	0.315	0.293
	42035	38960	134705	0.312	0.289
260.5	445739	408755	134276	3.32	3.04
	445437	408028	134215	3.32	3.04
	number of measurements, n			6	6
	correlation coefficient, r			0.99998	0.99994
	slope of calibration graph			0.0128	0.0117
	intercept (y-axis)			-0.00875	0.00282
	intercept (x-axis), µg/ml			0.69	-0.24

Table 4.6. Summary of results: semi-preparative resolution of trimeprazine enantiomers with on-line recovery.

fraction 1

run	recovery %	%e.e.	loss in flushing, %	throughput mg/hour
1	80	98	10	1.1
2	65	>98*	1	1.5
3	46	>98*	<1**	0.8
4	83	>98*	1	1.2
5	89	>98*	11	1.9

fraction 2

run	recovery %	%e.e.	loss in flushing, %	throughput mg/hour
1	35	92	25	0.5
2	38	90	24	0.7
3	37	92	16	0.6
4	62	92	28	0.9
5	54	92	60	1.2
6	85	92	<1**	1.5

* = optical impurity not detected

**= no loss detected

The optical purities of the peak 2 fractions were also generally lower (90 - 92% e.e. as opposed to 98% e.e. or higher for the peak 1 fractions), owing to the "tail" of the first eluting peak co-eluting with the second peak fraction. Considerable losses at the "flushing" stage of the procedure were observed in several cases. The mildest flushing procedure, (iv), using small amounts of water, followed by acetonitrile-water (10:90, v/v) was found to be effective at selectively removing the cyclodextrin from the recovery column. Very high recoveries were obtained when this procedure was employed.

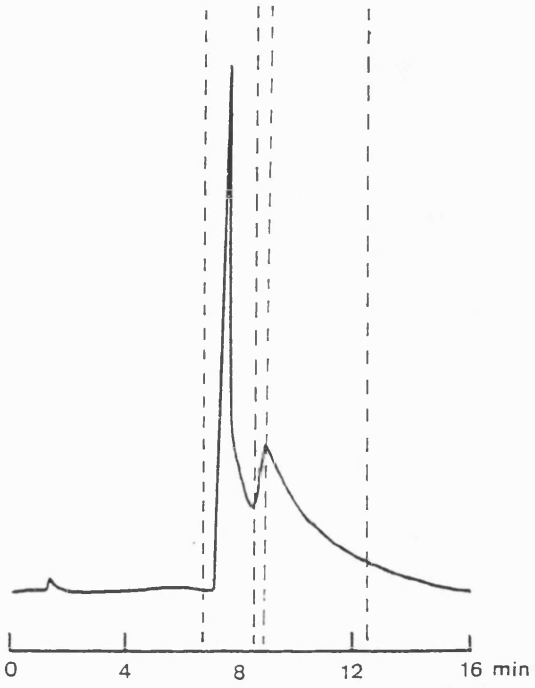


Figure 4.7. Semi-preparative resolution of trimeprazine enantiomers (1mg racemate on column). Column: SGE-100GLC4-C8-30/5. Mobile phase: acetonitrile - aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 9mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75ml/min. ISS valve switching points for recovery of enantiomers are also shown (dotted lines).

The resolution obtained in run 6 is illustrated in Figure 4.7, which also shows the valve

switching times employed for recovery of the enantiomer fractions. The optical purity of the fractions obtained in this run are illustrated in the chromatograms obtained on reinjection, Figure 4.8.

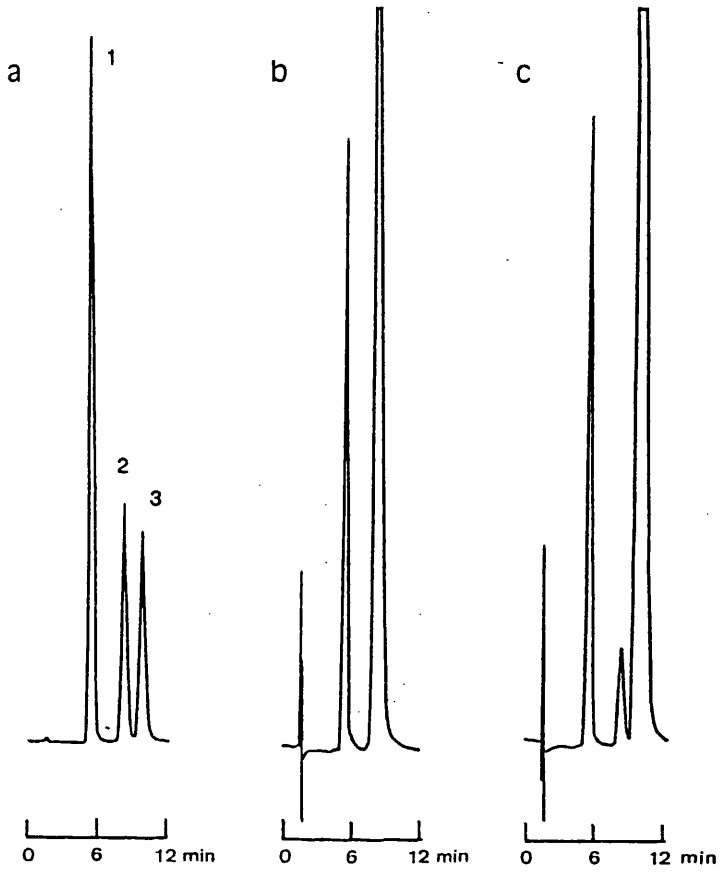


Figure 4.8. Analytical resolution of trimeprazine enantiomers, Conditions as Figure 4.7. Peak identities: (1) levomepromazine (internal standard), 0.25µg on col.; (2) and (3) trimeprazine enantiomers. Sample identities: (a) ±-trimeprazine tartrate 0.25µg on col. (calibration standard) (b) recovered trimeprazine peak 1 fraction (e.e. >98%) (c) recovered trimeprazine peak 2 fraction (e.e. 92%)

NMR analysis of the collected fractions from all six semi-preparative runs showed them to contain significant quantities of triethylamine, as shown by characteristic signals at 1.1ppm

(triplet) and 3.2ppm (quartet). Some of the fractions, particularly those from runs 2 to 5, showed the presence of ß-cyclodextrin, as evidenced by characteristic signals at 5.1ppm (doublet) and 3-4ppm (multiplets), indicating that the flush procedures used in these cases were not fully effective. The fractions from run 6, where a larger volume of weaker flush solvent had been employed, showed the presence of only trace quantities of ß-cyclodextrin, as illustrated in Figure 4.9.

The procedure was found to be robust and reproducible. Over the 4 hours of a 16-injection run, no significant changes in retention were observed, despite slight variation in laboratory temperature. No adjustment of valve switching times during the course of a run were therefore necessary, and the system could therefore be left to run unattended during the loading of the recovery columns. In principle, with the use of a suitable ternary gradient system to control the necessary solvent changes, a fraction collector to recover the enantiomers on elution from the recovery columns, and with microcomputer control, the whole system could be automated. This possibility was not explored, however, as the required equipment was not available.

While the optimised flushing procedure proved capable of producing the resolved enantiomers free from cyclodextrin, the problem of triethylamine contamination required further study. The use of a TFA-containing eluting solvent, while making for easy recovery of product from the eluting solvent, produced trimeprazine trifluoroacetate as product, which proved to be largely insoluble in water. The production of water-soluble salts (typically maleates, tartrates, or hydrochlorides) would be advantageous in analysis and for the potential end-user of resolved material.

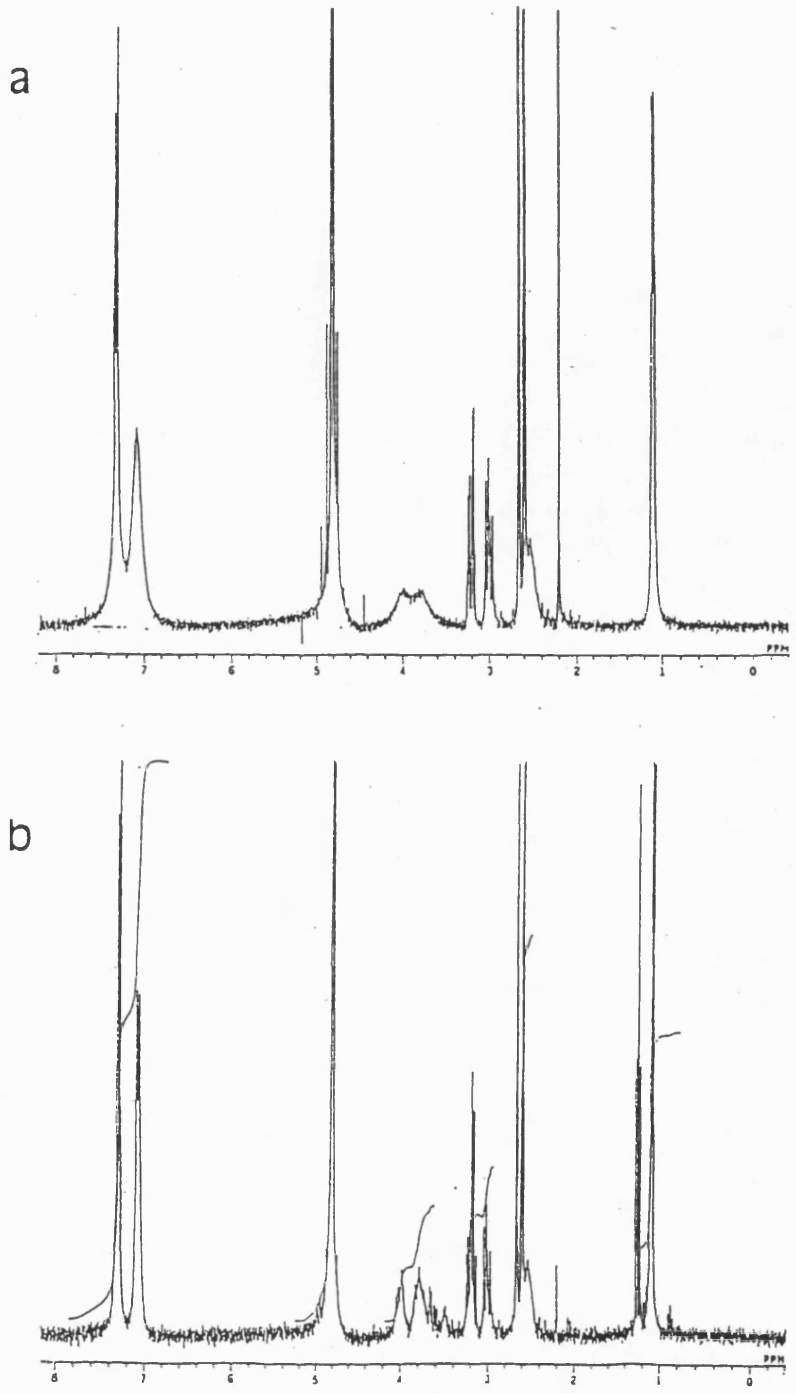


Figure 4.9. 270MHz ^{1}H-NMR spectra of trimeprazine trifluoroacetate. (a) racemate (prepared from racemic tartrate as described in 2.1.4.) (b) recovered peak 1 fraction from semi-preparative run 6.

4.2.5 Solvent extraction recovery procedure.

The use of a liquid-liquid extraction procedure to recover enantiomers from the chiral mobile phase following separation was investigated. The procedure involved adding TFA to the HPLC fraction, followed by extraction of the trifluoroacetate into ether, and evaporation of the ether layer to reclaim the crude trifluoroacetate. NMR analysis of the resulting product showed that the procedure was effective at removing ß-cyclodextrin from the product (owing to the very low solubility of ß-cyclodextrin in ether), but that substantial triethylamine contamination was still present. Presumably, the triethylamine trifluoroacetate was significantly extracted into ether.

A similar experiment with triethylamine-contaminated fractions from the on-line recovery procedure showed that solvent extraction was unable to significantly reduce the amount of triethylamine present. While this procedure might be employed as an alternative to the on-line approach for resolved enantiomers where no triethylamine in mobile phase was employed, it was not seen to offer any advantages in this particular case.

4.2.6 Use of polymeric recovery column.

The use of a polystyrene-divinylbenzene (Hamilton PRP-1) stationary phase for the recovery of trimeprazine was investigated. This material was found to have a higher capacity for trimeprazine than the Lichroprep RP18 material. 10.0mg per gram ±-trimeprazine tartrate could be loaded onto this material without significant breakthrough loss, compared to about 6mg per gram on Lichroprep RP18 under the same conditions.

The PRP-1 column was flushed with 10ml of acetonitrile - water (10:90, v/v), followed by 10ml acetonitrile - TFA- water (10:0.1:89.9, v/v); and the trimeprazine could be eluted at high recovery in 20ml acetonitrile - TFA - water (70:0.1:29.9, v/v). NMR analysis of the reclaimed trimeprazine showed that the procedure had been effective as before at removing

ß-cyclodextrin, but that triethylamine was present. This was somewhat surprising, since the lack of silanol groups on the polymeric material might be expected to lead to much lower affinity for triethylamine than on the silica-based RP18.

The flushing of triethylamine from the PRP-1 phase was further investigated in an experiment in which the column was equilibrated with the chiral mobile phase, and flushed with acetonitrile - water (10:90, v/v). Samples of the eluate were taken and analysed for triethylamine content by HPLC using on-line post-column ion-pair formation with 9,10-dimethoxyanthracenesulphonate (DAS), extraction into organic solvent, and fluorescence detection of ion-pairs in then organic phase. Figure 4.10 shows the variation in triethylamine content in the eluate with volume of flushing solvent passed through the column.

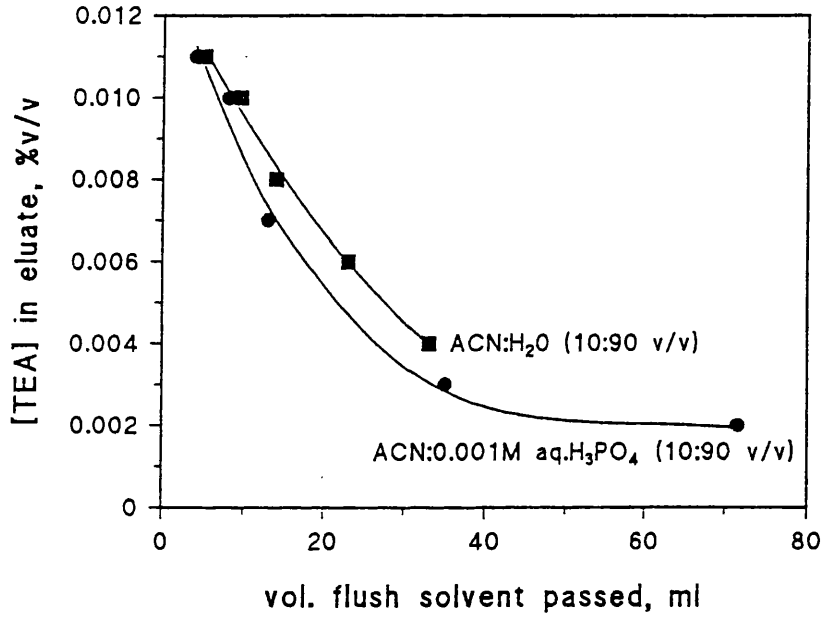


Figure 4.10. Triethylamine content of eluate from Hamilton PRP-1 (150x4.6mm) column (dead volume = 2.49ml). Column pre-equilibrated with acetonitrile - aq. triethylammonium acetate (0.8% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 13.5mg/ml beta-cyclodextrin hydrate and flushed with (a) acetonitrile - water (10:90, v/v) (b) acetonitrile - aq. H_3PO_4 (0.001M) (10:90, v/v)

It can be seen that substantial quantities of flush solvent were necessary to remove all the triethylamine from the column. The situation was slightly improved by using an acidified flushing solvent, indicating that the phase has lower retentivity for triethylamine in protonated form.

The higher capacity of the PRP-1 phase would provide advantages in its use as a recovery column material for on-line recovery of trimeprazine enantiomers, but the recovery procedure using this column was unable to produce recovered material free from triethylamine.

4.2.7 Modification of recovery column loading

More thorough flushing of the PRP-1 phase was made possible by reducing the loading to 4.1mg per gram. A 100x10mm column could then be flushed with 360ml of acetonitrile-water (10:90, v/v) (about 65 column volumes), and the trimeprazine eluted with 50ml of methanol. The NMR spectrum of the product is shown in Figure 4.11. This shows that the product under these conditions was almost completely free from triethylamine and ß-cyclodextrin. Comparison with a trimeprazine sample "spiked" with triethylamine and ß-cyclodextrin suggested that the product contained considerably less than 0.2 mol% ß-cyclodextrin and 2 mol% triethylamine. No loss of trimeprazine recovery occurred during the flushing procedure. This was checked by addition of tartaric acid as internal standard to the product before NMR analysis.

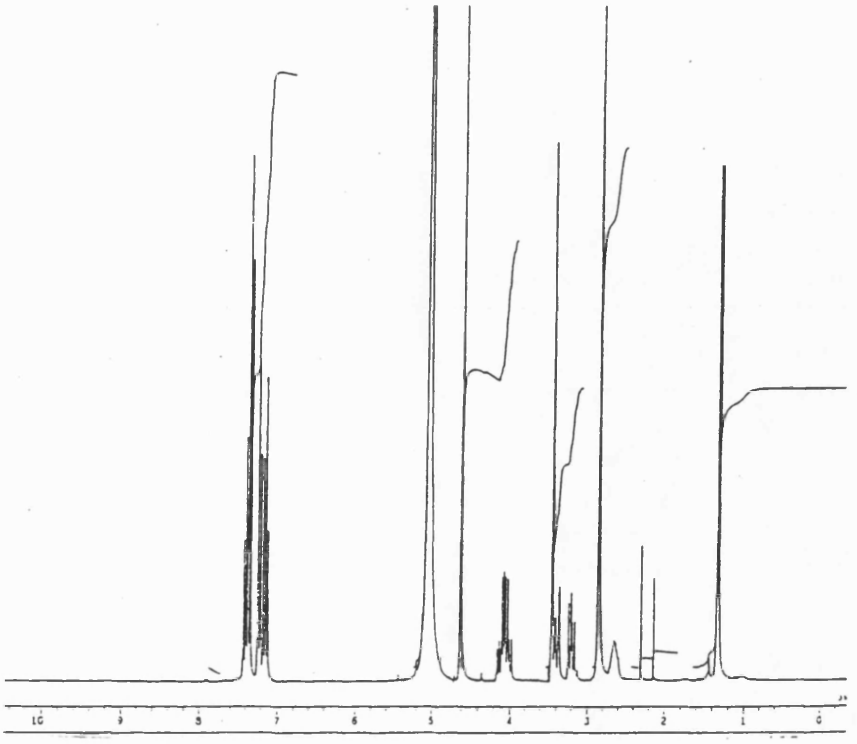


Figure 4.11 ^{1}H-NMR spectrum of trimeprazine recovered from PRP-1. Recovery procedure as text. 2 mol. equiv. (+)-tartaric acid added as int. std. Solvent: CD_3OD.

4.3 Thromboxane antagonists

4.3.1 Effect of loading on resolution of ±-TA1

The effect of column loading on resolution of TA1 enantiomers is illustrated in Table 4.7. Near-baseline resolution was observed up to 1mg racemate on column, due to the high selectivity (α = 1.6 at low column loading) induced by the eluent ß-cyclodextrin for this racemate. It was therefore anticipated that semi-preparative resolution of enantiomers of TA1 could easily be achieved by this approach.

4.3.2 On-line recovery of TA1 enantiomers following chiral separation.

On-line recovery of TA1 enantiomers was accomplished by the same principle as used for trimeprazine, using somewhat different instrumentation. Four complete semi-preparative runs were carried out for TA1, under conditions described in table 4.8. 1mg of racemate was introduced to the column at each injection.

The low solubility of ±-TA1 (about 4mg/ml) in the mobile phases used presented difficulties. To overcome these, samples for injection were dissolved in 50% DMSO/ 50% mobile phase, in which a concentration of 10mg/ml could be achieved. DMSO was used in preference to other solvents as it did not cause precipitation of ß-cyclodextrin from the eluent.

A typical chromatogram obtained at 1mg loading is shown in Figure 4.12, which also shows the recovery system valve switching times used.

Table 4.7. Effect of column loading and beta-cyclodextrin concentration on resolution of TA1 enantiomers. Column: SGE-100GLC4-C8-30/5 (100 x 4mm), containing 0.77g packing material. Mobile Phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v). Injection volume: 100µl.

eluent [beta-CD] mg/ml	racemate loading mg on col	k'_1	α	R_S	%CRF
15	0.015	14.5	1.56	3.8	100
	0.045	13.5	1.57	2.5	100
	0.140	12.4	1.56	2.8	100
	0.420	9.7		1.9	100
	0.69	9.0		1.5	100
	1.03	8.0			95
18	0.015	11.8	1.58	3.0	100
	0.045	10.9	1.56	2.2	100
	0.14	9.5	1.58	1.8	100
	0.42	7.8		1.7	99
	0.69	7.0			94
	1.03	6.3			89
21	0.015	10.4	1.61	3.1	100
	0.045	9.8	1.62	2.3	100
	0.14	8.8	1.63	1.8	100
	0.42	7.5			100
	0.69	6.6			96
	1.03	5.8			90

Table 4.8. Summary of semi-preparative procedures for resolution of TA1 enantiomers.

Run	eluent [BetaCD] mg/ml	flush procedure	loading, mg/enantiomer
1	15	1	5.8
2	18	2	6.0
3	21	3	6.0
4	21	4	5.5

Flush procedures:

(1): 5ml x acetonitrile - water (10:90, v/v)

(2): 5ml x TFA- water (0.2:99.8, v/v) followed by
5ml x acetonitrile -TFA - water (10:0.2:89.8, v/v)

(3): 10ml x acetonitrile - TFA - water (10:0.2:89.8, v/v)

(4): 10ml x acetonitrile - TFA - water (15:0.2:84.8, v/v)

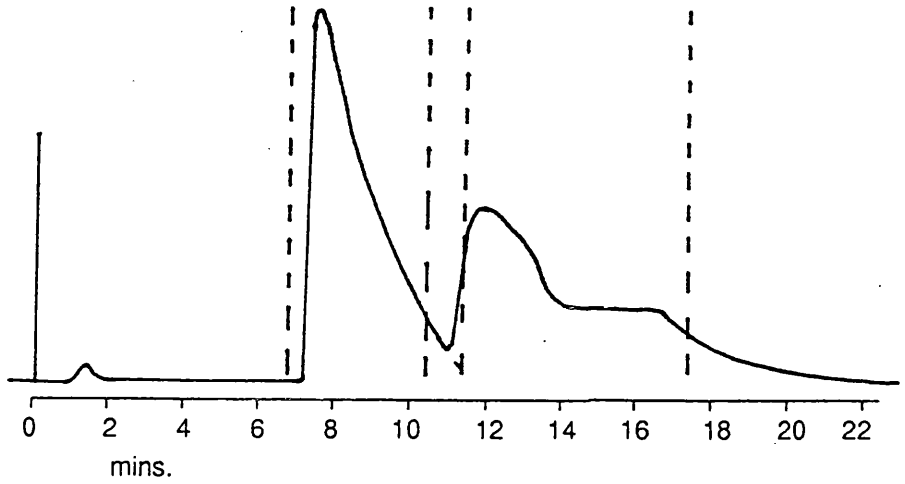


Figure 4.12. Resolution of TA1 enantiomers on SGE-100GLC4-C8-30/5 column. Eluent: ACN - aq. phosphate buffer (0.05M, pH7) (10:90 v/v) containing 21mg/ml ß-cyclodextrin hydrate. Sample: ±-TA1 (10mg/ml in DMSO - eluent (50:50 v/v)). Injection volume: 100µl. Detection: UV 290nm. Dotted lines show recovery system valve switching times. shown by dotted lines.

10-12 racemate injections were made per run before the recovery columns were flushed and the resolved enantiomers eluted and recovered. Eluates from the recovery columns were collected at both the "flushing" and elution stages, so that any loss of recovery could be accounted for. The "loading" part of the procedure could, in principle, be carried out unattended (as it had been for trimeprazine). In practice, however, manual injections were made, as the Varian autosampler tended to waste large amounts of sample in purging the loop at each injection.

The fractions were assayed for recovery and optical purity by re-injection into the chromatograph under the same conditions as above. m-Nitroaniline was used as an internal standard, and a calibration graph was constructed using peak height ratios. Typical calibration data is shown in Table 4.9. The semi-preparative results obtained are summarised in Table 4.10, and typical optical purities illustrated in Figure 4.13.

Table 4.9. Calibration data from chiral TA1 assay. Column: SGE100GLC4-C8-30/5. Mobile phase: acetonitrile - phosphate buffer (0.05M, pH 7) (10:90, v/v) containing 18mg/ml beta-cyclodextrin hydrate. Internal standard: m-nitroaniline. Injection volume: 20µl. Detection: UV 254nm. Integration: Varian data station.

[TA1],	peak heights			peak height ratios	
µg/ml/enantiomer	TA1		int.std.		
	peak 1	peak 2		peak 1	peak 2
26	349	233	8502	0.041	0.027
	337	238	8776	0.038	0.027
	356	239	8721	0.041	0.027
128	2047	1410	8798	0.233	0.160
	2036	1389	8734	0.233	0.159
	2035	1392	8750	0.233	0.159
638	10326	6902	8522	1.212	0.810
	10644	7091	8776	1.213	0.808
	10610	7079	8688	1.221	0.815
	number of measurements, n			9	9
	correlation coefficient, r			>0.9999	>0.9999
	slope			0.00192	0.00127
	intercept (y-axis)			-0.0087	-0.0019
	intercept (x-axis), µg/ml			4.5	1.5

Table 4.10. Summary of results: semi-preparative resolution of TA1 enantiomers with on-line recovery

run	fraction	recovery %	e.e., %	loss in flushing, %	throughput mg/hour
1	1	99	98.4	23	1.35
	2	70	99.2	15	0.96
2	1	87	>99*	2	1.37
	2	72	98.4	15	1.13
3	1	85	99.1	2	1.4
	2	77	98.4	**	1.3
4	1	51	90.3	3	0.9
	2	72	99	**	1.3

* = no optical impurity detected

** = not determined

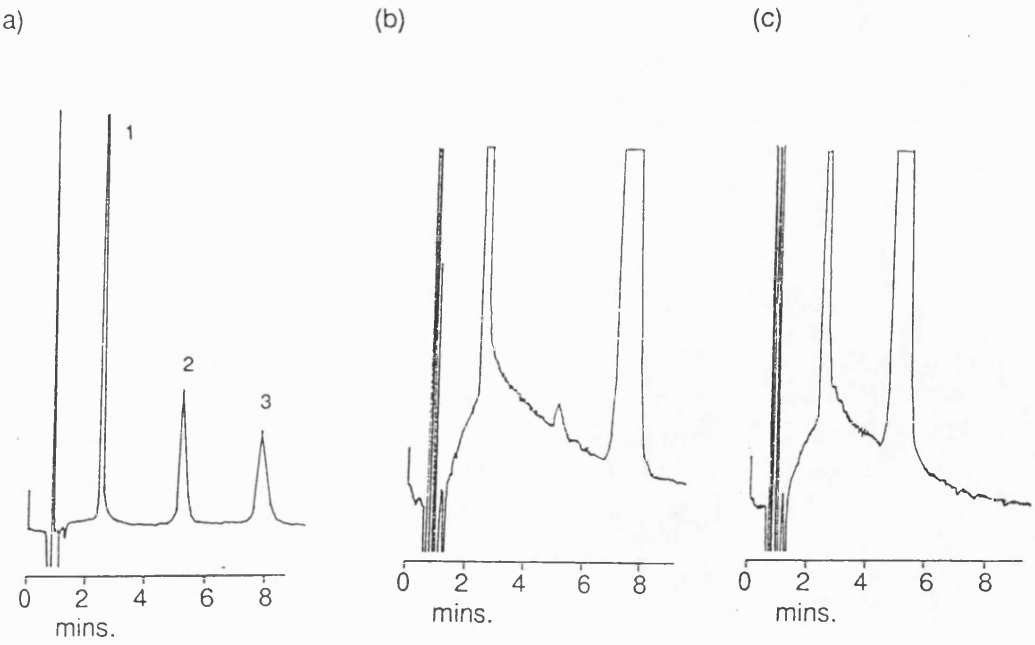


Figure 4.13. Analytical resolution of TA1 enantiomers on SGE-100GLC4-C8-30/5. Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v) containing 21mg/ml ß-cyclodextrin hydrate. Flow rate: 0.75ml/min. Injection volume: 20µl. Detection: UV 254nm. Peak identities: (1) m-nitroaniline (internal standard, 0.1mg/ml). (2) and (3): TA1 enantiomers. (a) ±TA1 standard, 0.256mg/ml, ATTEN 8 (b) peak 2 semi-prep fraction, ATTEN 1 (c) peak 1 semi-prep fraction, ATTEN 1.

The throughputs obtained here (1 - 1.5mg of each enantiomer per hour) are comparable to those obtained for trimeprazine. In general, however, higher optical purities (>99% for both peaks) were obtained in this case, reflecting the higher enantioselectivity. Throughput could be increased further by increasing the column loading, at the expense of optical purity. In general, increasing the cyclodextrin concentration in the eluent increased throughput, although not markedly, owing to shorter run time.

The flush procedure was not effective in all cases. In run 1, loss of TA1 from the recovery columns occurred during the flushing stage. In subsequent runs, TFA was incorporated into the flush solvent in order to suppress carboxylic acid ionisation and hence improve

retentivity of TA1 on the recovery column. This was effective, as shown by the much smaller losses on flushing sustained. NMR analysis of the recovered enantiomers from runs 2 and 3 revealed them to contain significant quantities of ß-cyclodextrin, as exemplified in Figure 4.14b. The eluting strength of the flush solvent was increased in run 4. Recovered enantiomers from this run showed less cyclodextrin contamination, as shown in Figure 4.14c. This was quantified from NMR integrals to be up to 3 mol% in fraction 1, and 2 mol% in fraction 2. It is clear that more prolonged flushing should be carried out to reduce this contamination.

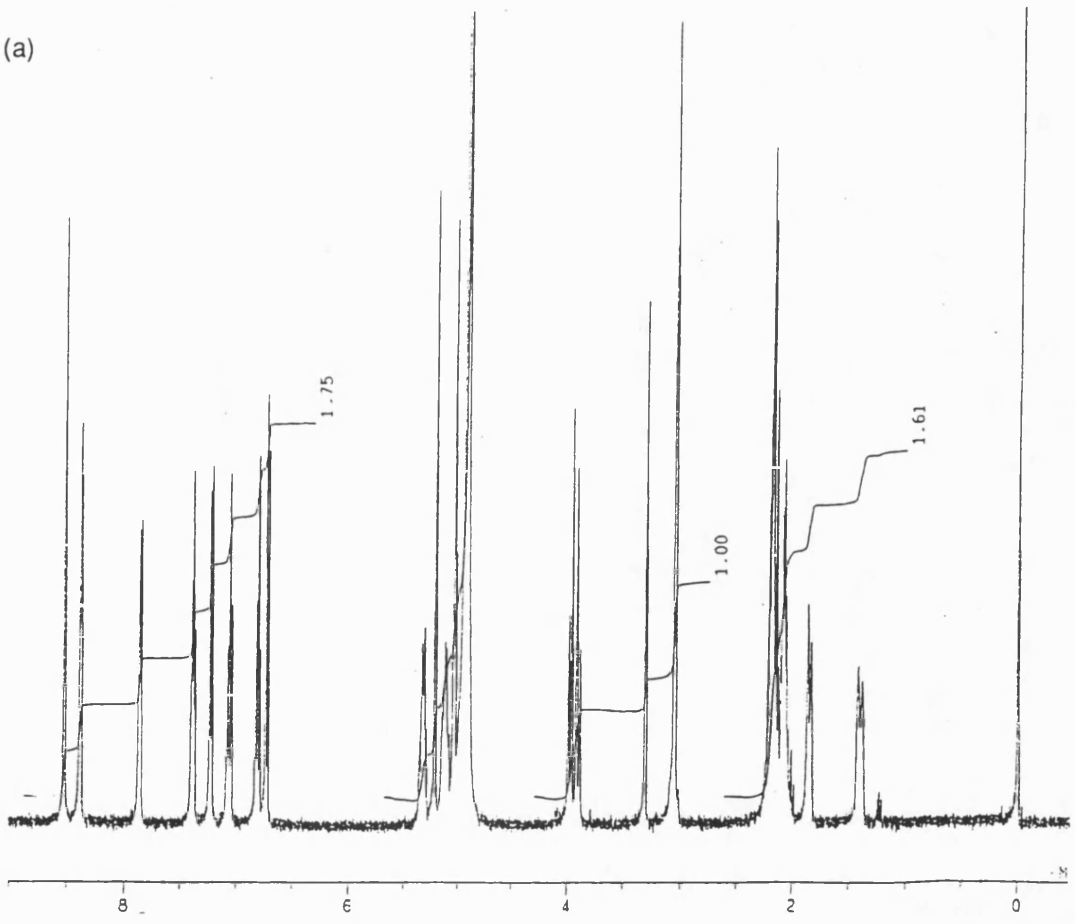
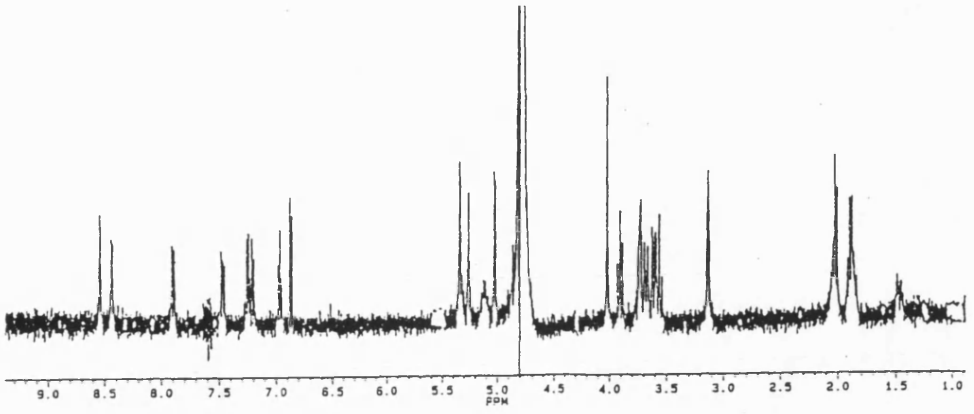
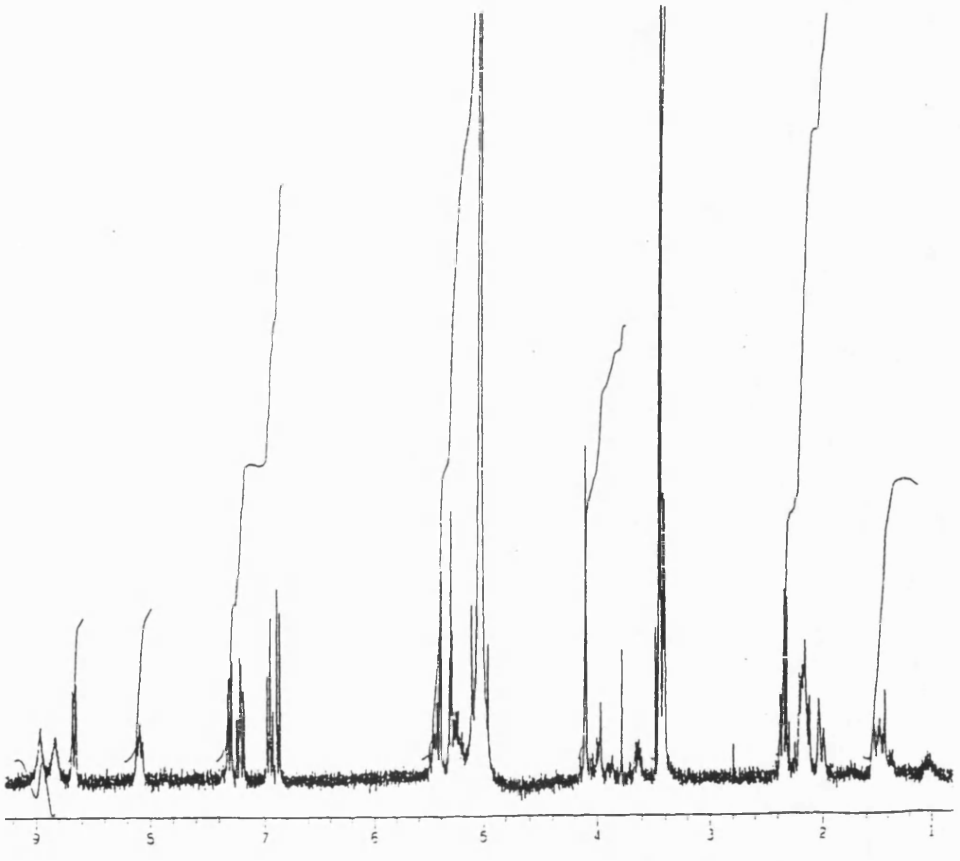


Figure 4.14. ^{1}H-NMR spectra of TA1 in CD_3OD. (a) racemate (400MHz spectrum) (b) peak 1 fraction from run 2 (400MHz spectrum) (c) peak 1 fraction from run 4 (270MHz spectrum)

Fig. 4.14(b)



(c)



Methanol was used as the eluting solvent in this case owing to the higher solubility of TA1 in methanol compared to acetonitrile. The higher volatility of methanol compared to acetonitrile and acetonitrile - water mixtures facilitated recovery of the enantiomers from the elution solvent without heating.

4.3.3 Semi-preparative resolution of TA12 enantiomers

TA12 was resolved somewhat less easily than TA1, with an α value of around 1.2, and near-baseline resolution on an SGE-100GLC4-C8-30/5 column with an eluent consisting of acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90,v/v) containing 20mg/ml ß-cyclodextrin hydrate. Under these conditions, the resolution shown in Figure 4.15 was achieved at 1mg column loading, This degree of resolution was deemed adequate.

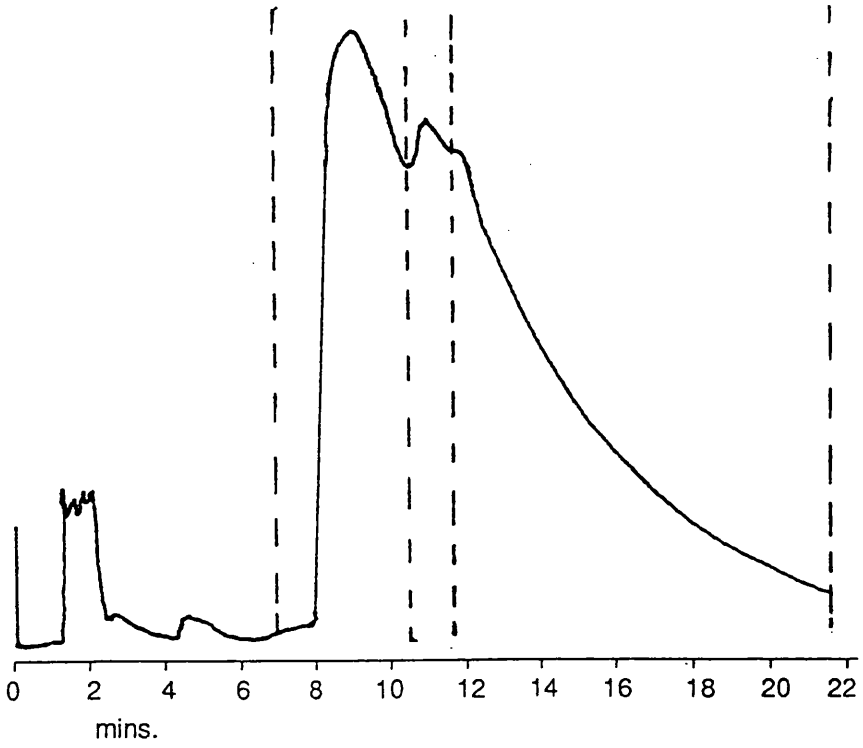


Figure 4.15. Semi-preparative resolution of TA12 enantiomers. Conditions as text. Detection UV 290nm. Valve switching points for recovery system are also indicated.

The semi-preparative procedure employed was very similar to that used for TA1. Eleven 1mg injections of racemate (100µl x 10mg/ml in 50% DMSO / 50% mobile phase) were made, before the recovery columns were flushed and the fractions eluted.

The recovered fractions were assayed for recovery and optical purity using a Cyclobond I column, as better resolution per unit time could be achieved by this approach than by using ß-cyclodextrin-containing eluents with the columns available. A calibration graph was constructed using peak height ratios, with m-nitroaniline as internal standard. Calibration data is given in Table 4.11, and chromatograms illustrating the optical purities obtained are shown in Figure 4.16. Recovery and optical purity data is summarised in Table 4.12. The NMR spectra of the fractions are shown in Figure 4.17.

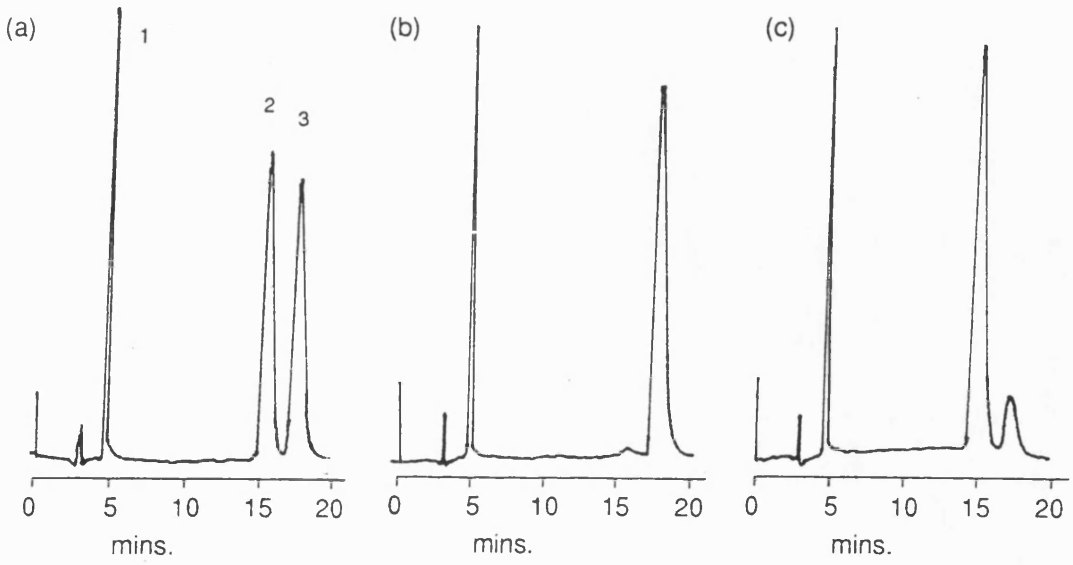


Figure 4.16. Analytical resolution of TA12 enantiomers on Cyclobond I (250 x 4.6mm). Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v). Detection UV 270nm. Peak identities: (1): Internal standard (m-nitroaniline), 40ng on col. (2) and (3): TA12 enantiomers. (a) standard ±TA12, 1.08µg on col. (b) semi-prep peak 1 fraction (c) semi-prep peak 2 fraction. Note that elution order of enantiomers is reversed relative to cyclodextrin mobile phase system.

Table 4.11. Calibration data for chiral TA12 assay. Column: Cyclobond I (250 x 4.6mm). Mobile phase: acetonitrile - aq. phosphate buffer (0.05M, pH 7) (10:90, v/v). Internal standard: m-nitroaniline (0.1mg/ml). Injection volume: 20µl. Detection: UV 270nm. Integration: Varian data station.

[TA12],	peak heights			peak height ratios	
µg/ml/enantiomer	TA12		Int. std.		
	peak 1	peak 2		peak 1	peak 2
10	1.5mm*	2mm*	91mm*	0.016	0.022
	1.5mm*	1.5mm*	90mm*	0.017	0.017
30	115	120	2251	0.051	0.053
	137	135	2346	0.058	0.058
89	419	390	2390	0.175	0.163
	413	366	1362	0.175	0.155
266	1160	1008	2344	0.495	0.430
	1290	1122	2580	0.500	0.435
798	2919	2492	2003	1.457	1.245
	3570	2986	2116	1.687	1.411
	number of measurements, n			10	10
	correlation coefficient, r			0.9959	0.9970
	slope			0.00197	0.00166
	intercept (y-axis)			-0.007	0.0038
	intercept (x-axis), µg/ml			4	-2

* = peak heights measured manually

Table 4.12. Summary of results: semi-preparative resolution of TA12 enantiomers with on-line recovery. (n.d. = not detected).

fraction	recovery %	e.e., %	loss in flushing, %	throughput mg/hour	beta-CD content, mol%
1	61	96.3	n.d.	0.74	1.0
2	72	87.6	n.d.	0.87	0.4

(a)

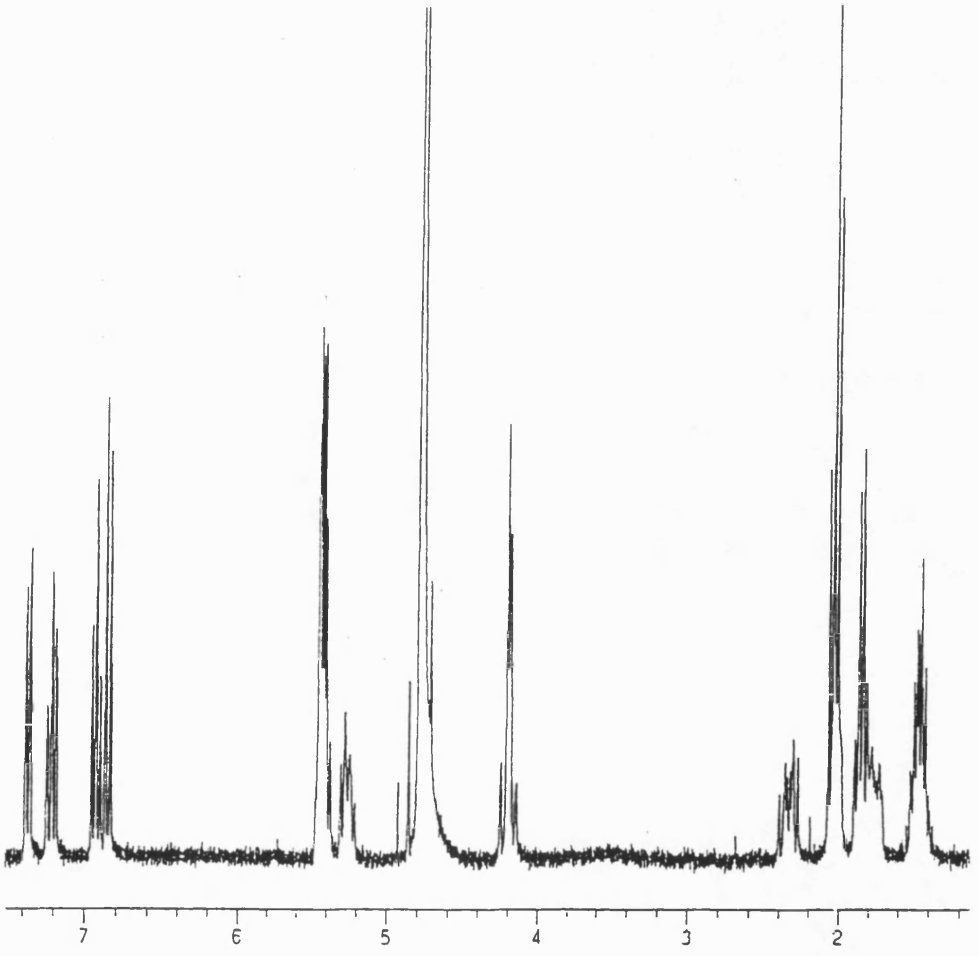
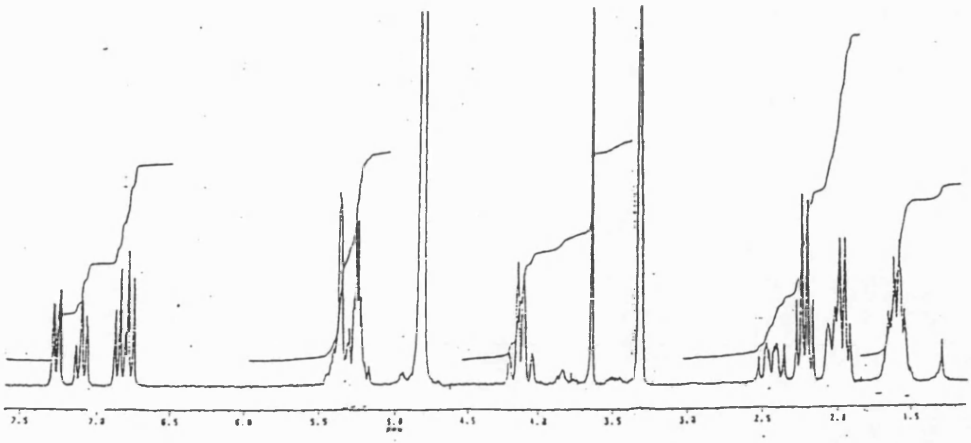
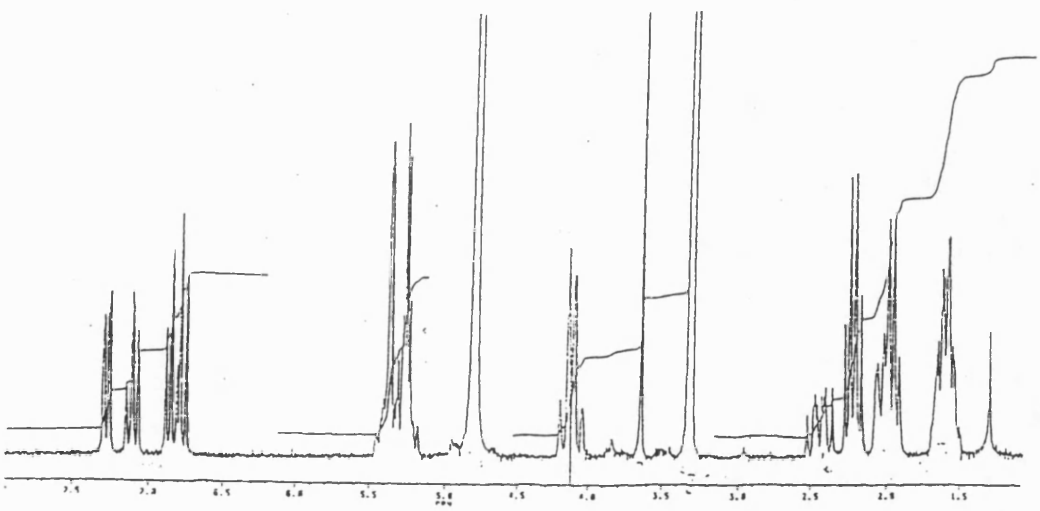


Figure 4.17. ^{1}H-NMR spectra of TA12. (a) 270 MHz spectrum of ±TA12 in D_2O. (b) 400MHz spectrum of semi-prep peak 1 fraction in CD_3OD. (c) 400MHz spectrum of semi-prep peak 2 fraction in CD_3OD.

Fig. 4.17(b)



(c)



Throughput for TA12 was somehat lower than that achieved for TA1 or trimeprazine, owing to low recovery and longer run time. The optical purities achived were also lower, and a "second pass" through the system might be required in this case to yield the degree of optical purity required for pharmacological testing of the enantiomers.

The recovery procedure was seen to be satisfactory. The "flushing" stage resulted in no loss of TA12 recovery, while only trace amounts of ß-cyclodextrin appeared in the product. Again, longer flushing would undoubtedly result in lower cyclodextrin contamination (as was demonstrated for trimeprazine).

4.4 Brompheniramine

4.4.1 Optimisation of brompheniramine enantiomer separation.

The effect of on-column loading on resolution of brompheniramine enantiomers on a Spherisorb S5CN column (250 x 4.6mm) using various ß-cyclodextrin-containing mobile phases is shown in Table 4.13. This column was chosen on the basis of its relatively low retentivity (allowing the use of low concentrations of organic modifiers in mobile phases), and also because a 10mm i.d. column packed with this material was available, allowing larger-scale separations.

Highest selectivity was observed when using a mobile phase containing 5% DMSO v/v, and 20mg/ml ß-cyclodextrin hydrate. Highest resolution at low column loading was observed using a similar eluent with a lower cyclodextrin content, the reduced cyclodextrin content causing increased retention and hence improved resolution. However, at 1mg column loading, highest resolution was obtained using an eluent containing 5% methanol (v/v) and 11mg/ml ß-cyclodextrin hydrate. The resolution obtained under these conditions was deemed adequate for semi-preparative separation of the enantiomers.

Increasing pH from 4 to 7.4 was found to increase retention, but had little effect on selectivity or resolution. Triethylamine was added to all eluents in order to reduce peak tailing. Variation in injection volume, over the range 20 to 200µl (at constant 1mg loading) was found to have no observable effect on resolution.

Table 4.13. Effect of mobile phase composition and column loading on resolution of brompheniramine enantiomers on a Spherisorb S5CN (250 x 4.6mm) column (containing 2.5g packing material). All mobile phases contain 0.8% triethylamine, v/v and acetic acid to pH 4. Inj. vol. 20µl

organic modifier, v/v	[BetaCD], mg/ml	k'_1	2µg on col. α	%CRF	1mg on col. %CRF
5% CH_3CN	16	0.9	1.14	55	42
10% CH_3CN	20	0.8	1.12	39	low
3% CH_3OH	11	1.5	1.14	48	36
5% CH_3OH	11	1.7	1.14	43	50
5% DMSO	11	1.6	1.12	57	18
5% DMSO	20	1.1	1.15	49	30
5% EtOH	20	0.9	1.12	42	low

In order to increase throughput, further studies were carried out on a 250mm x 10mm i.d. column packed with the same material. This column contained 4.7 times more packing material than the 4.6mm column above. However, the flow rate employed was limited to 3.5ml/min., due to high back-pressure. The effect of loading on resolution on this 10mm i.d. column is illustrated in Figure 4.18.

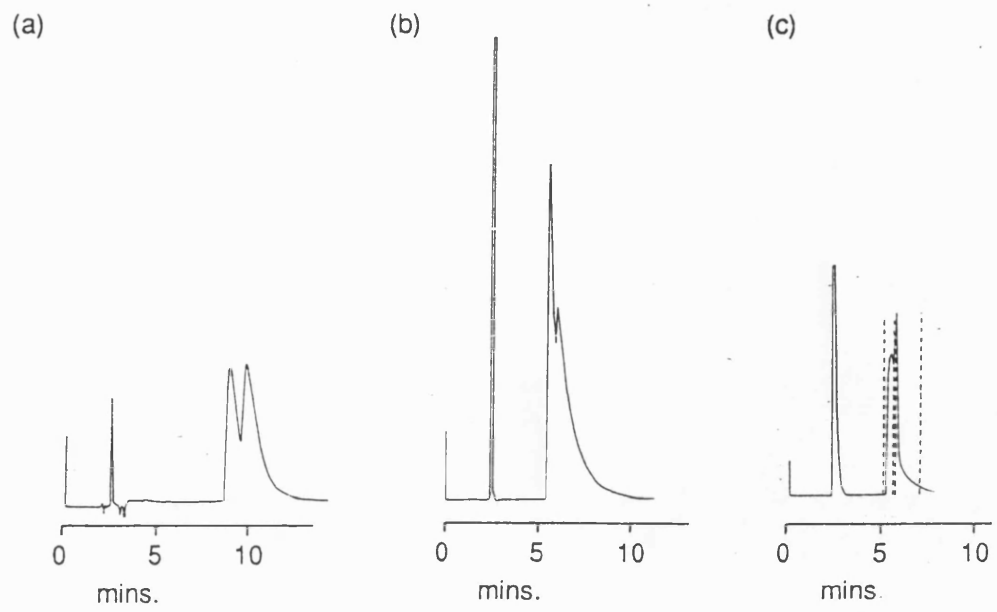


Figure 4.18. Resolution of brompheniramine enantiomers on an S5CN 250 x 10mm i.d. column, containing 11.8g packing material. Mobile phase: methanol - aq. triethylammonium acetate (0.85% TEA v/v, acetic acid to pH 4) (5:95 v/v) containing 12mg/ml ß-cyclodextrin hydrate. Flow rate: 3.5ml/min. Injection solution: ±-brompheniramine maleate in mobile phase. Loading: (a) 10µg on col. (10µl x 1mg/ml) (b) 2.5mg on col. (50µl x 50mg/ml) (c) 5mg on col. (100µl x 50mg/ml). Detection: UV (a) 254nm 0.1AUFS (b) 280nm 2.0AUFS (c) 285nm 2.0AUFS. Fraction collection switching points are shown (dotted lines).

The change in peak shapes as column loading was increased is of interest. Initially, increases in column loading caused marked increases in the tailing of peak 2, resulting in peak 1 being taller than peak 2. However, above 2.5mg on column, the tailing of peak 1 became more severe, so that peak 2 appeared taller. The second situation is clearly disadvantageous with respect to the collection of peak 2 at high optical purity. However, overall throughput might be maximised by using a high column loading, with a second pass through the system used to further purify the initial fractions. Thus 5mg on column was the loading used in semi-prep runs.

4.4.2. Optimisation of recovery procedure

The capacities of Hamilton PRP1 (12-20µm) and Lichroprep RP18 (25-40µm) materials for ±-brompheniramine maleate, loaded at 1mg/ml in methanol - aqueous triethylammonium acetate (0.84% TEA v/v, acetic acid to pH 4) (5:95, v/v) containing 12mg/ml ß-cyclodextrin hydrate were measured in off-line "breakthrough" experiments. The capacities of columns packed with the two material were found to be 3.8mg per gram packing material (PRP1) and 2.8mg per gram packing material (RP18).

In a subsequent experiment, 2.4mg per gram packing material ±-brompheniramine maleate was loaded onto PRP-1. The column was flushed with 30 column volumes (164ml) of methanol-water (5:95 v/v), and the brompheniramine eluted in 5 column volumes of methanol (25ml). The brompheniramine was recovered at more than 90% yield containing less than 0.1 mol% ß-cyclodextrin or 1 mol% triethylamine (as determined by NMR using maleic acid as internal standard.) When Lichroprep RP18 was loaded with brompheniramine under the same conditions, 46% of the brompheniramine was lost during the flushing of the column. The remaining brompheniramine was free from cyclodextrin or buffer components.

It was clear from these experiments that Hamilton PRP-1 was the more appropriate choice of material for on-line recovery of brompheniramine following chiral separation, owing to its higher capacity.

4.4.3 Semi-preparative resolution of brompheniramine enantiomers with on-line recovery

Semi-preparative resolution of brompheniramine enantiomers was accomplished under the optimised conditions described above, with on-line recovery achieved using Hamilton PRP-1. Repeat injections of racemate (22 x 5mg) were made into the 250 x 10mm S5CN column, giving a loading of 55mg onto each of the recovery columns. These were then

each flushed with 140ml (25 column volumes) of methanol - water (5:95 v/v) before the brompheniramine fractions were eluted in methanol. The fractions were introduced to the system a second time, under identical conditions, to increase their optical purity.

The optical purities and recoveries obtained by HPLC chiral analysis of the fractions at each stage are shown in table 4.14, and illustrated in figure 4.19. The more efficient and retentive C6 column gave near-baseline resolution of the enantiomers under analytical conditions. Overall recoveries were also checked by NMR, using maleic acid as internal standard. Good agreement was obtained between the two techniques.

Table 4.14. Summary of results: semi-preparative resolution of brompheniramine enantiomers with on-line recovery. Recovery and optical purity determined by HPLC. Recovery and chemical purity determined by NMR. Throughput values derived from recoveries determined by HPLC.

Peak	Recovery, %	e.e., %	throughput, mg/hour	beta-CD content, mol%	TEA content, mol%
After 1st pass:					
1	68	82.2	11.9		
2	72	75.8	12.8		
After 2nd pass:					
1	49.7 (HPLC) 48.3 (NMR)	95.4	4.5	<0.2*	<1*
2	35.1 (HPLC) 39.2 (NMR)	88.0	3.2	0.4	<1*

* = not detected in product.

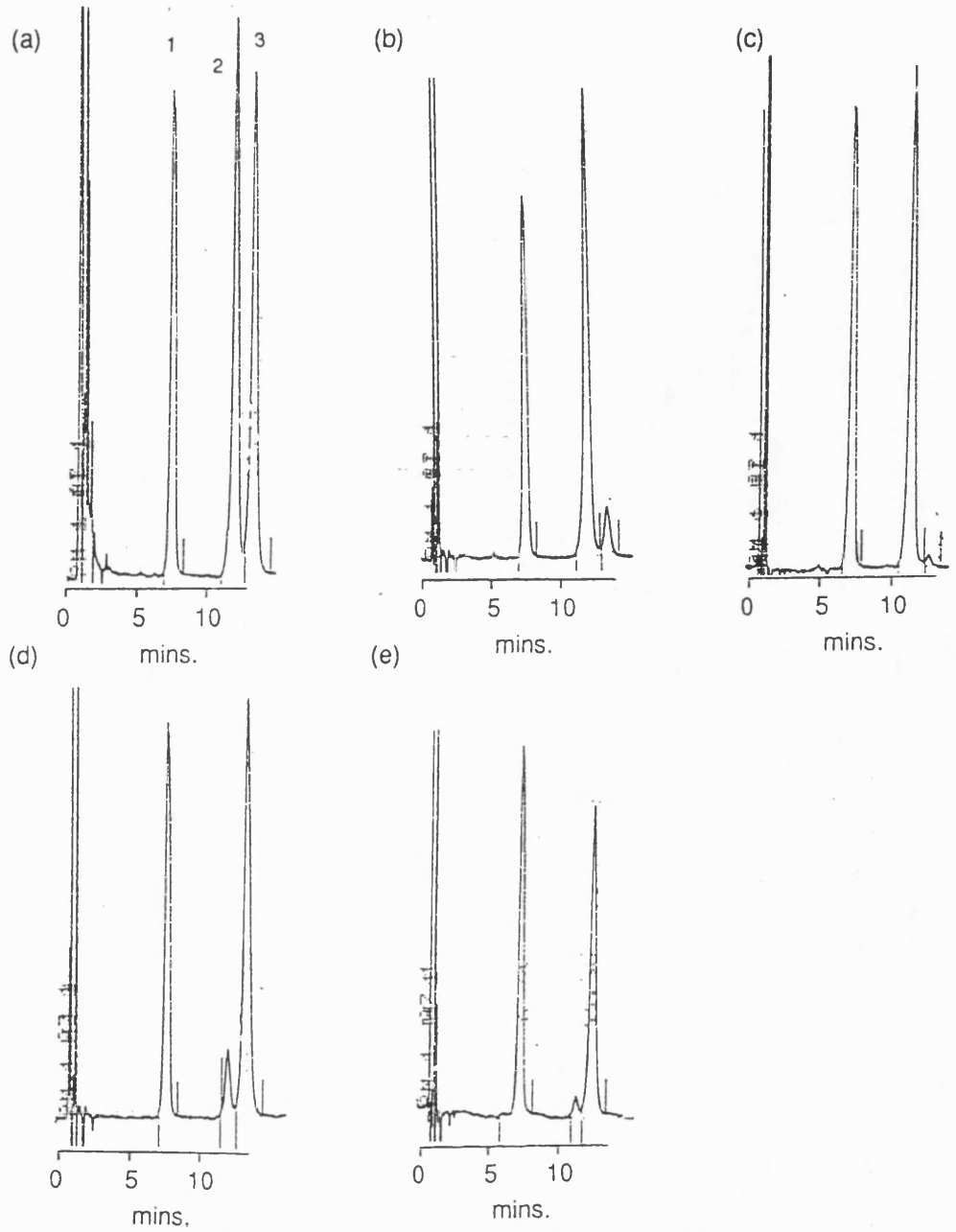


Figure 4.19. Analytical resolution of brompheniramine enantiomers on S5C6 (150 x 3mm) column. Eluent: acetonitrile - aq. triethylammonium acetate (0.89% TEA v/v, acetic acid to pH 4) (10:90, v/v) containing 20mg/ml ß-cyclodextrin hydrate. Flow rate: 1ml/min. Inj. vol.: 10µl. Detection: UV 254nm. Peak identities: (1) ±-pheniramine (int. std., 1µg on col.) (2) and (3) brompheniramine enantiomers. (a) ±-brompheniramine maleate standard, 3.5µg on col. (b) peak 1 fraction after 1st pass (c) peak 1 fraction after 2nd pass (d) peak 2 fraction after 1st pass (e) peak 2 fraction after 2nd pass.

Figure 4.20 illustrates the chemical purity of the fractions. They were found to contain up to 0.4 mol% ß-cyclodextrin, but no triethylamine was detected by NMR.

No brompheniramine was detected in the eluate from the columns on flushing, suggesting that less than 1% of the brompheniramine was lost during the flushing stages of the recovery procedure. The overall recoveries obtained therefore reflect the diversion of optically impure material to waste between the two collected fractions. The greater loss of peak 2 reflects the difficulty in collecting this peak pure due to the tailing of peak 1. If recovery was critical, less material could be diverted to waste. More passes through the system would then be needed to produce a given degree of optical purity. For this reason, recovery must be "traded off" against throughput or purity.

(a)

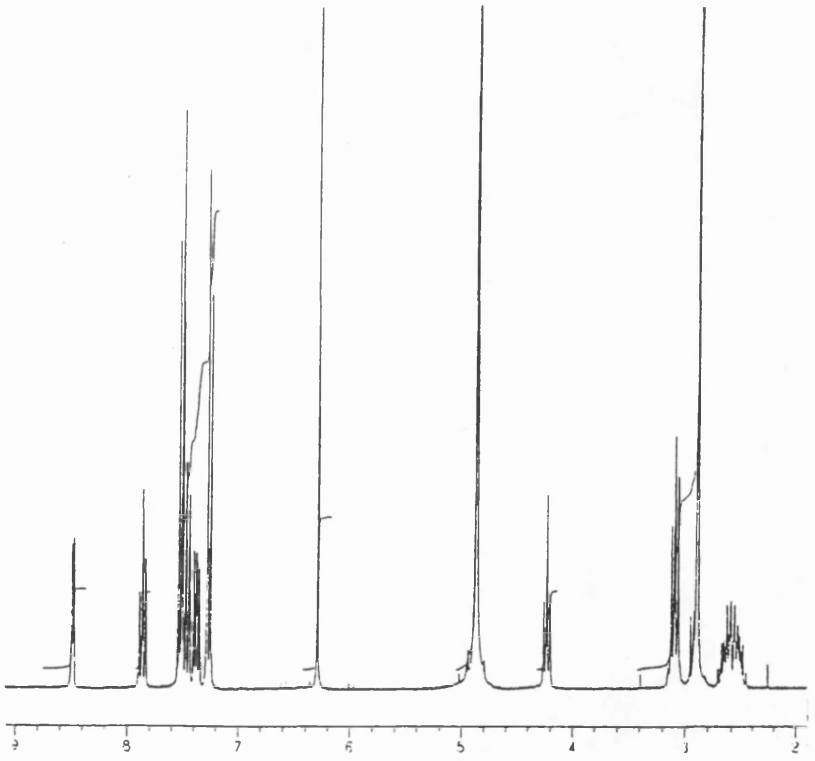
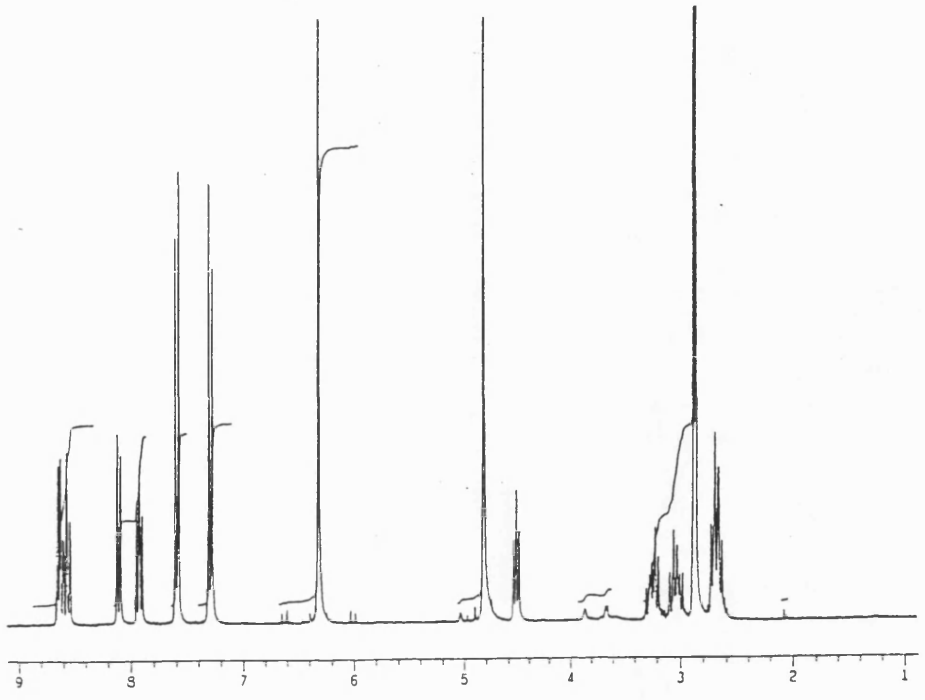
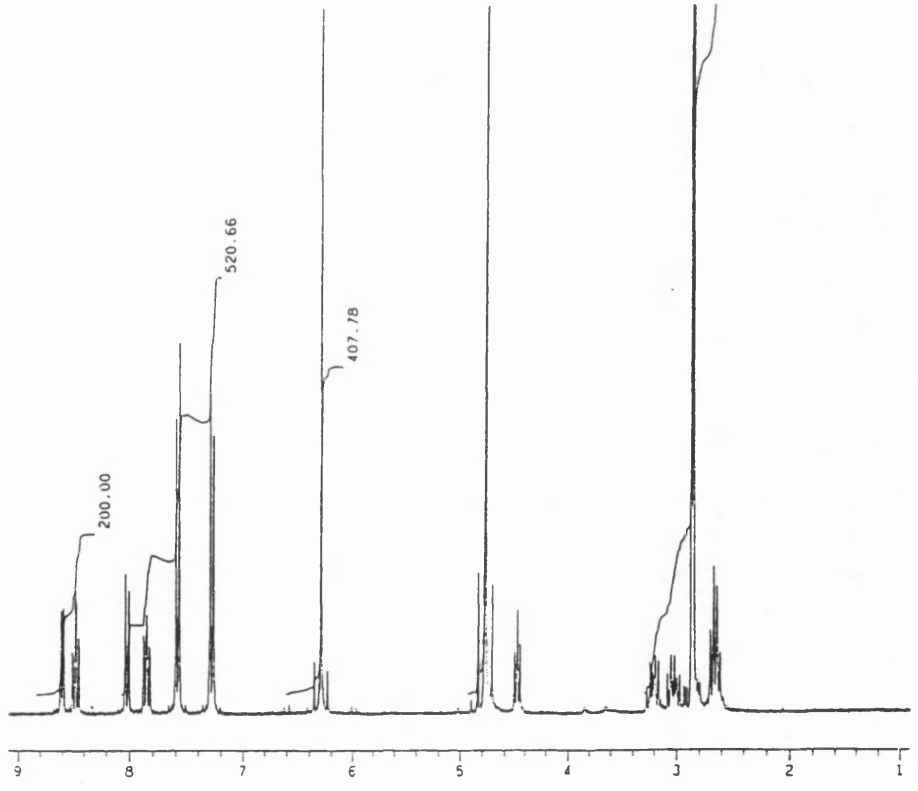


Figure 4.20. 270MHz ^{1}H-NMR spectra of brompheniramine maleate in D_2O. (a) racemate (b) peak 1 fraction after 2nd pass (c) peak 2 fraction after 2nd pass.

Fig. 4.20(b)



(c)



The optical rotation of the fractions (as free base) in DMF was determined. The peak 1 fraction gave a positive optical rotation (+0.18^{o} from an approx. 8mg/ml solution). The peak 2 fraction gave a negative optical rotation (-0.17^{o} from an approx. 6mg/ml solution). These results are consistent with those of Mercer (1989), who found that (-)-brompheniramine eluted before the (+)-enantiomer on a Cyclobond I column, which might be expected to give reversed elution order compared to the ß-cyclodextrin mobile phase employed here.

Further confirmation of chiral separation was provided by the NMR spectra of the fractions in the presence of beta-cyclodextrin. As reported by Casy and Mercer (1988), the addition of beta-cyclodextrin to racemic brompheniramine causes duplication of NMR signals. On addition of beta-cyclodextrin to the fractions resolved as described above, no duplication of signals was observed. On mixing the fractions to re-form a racemate, duplication of some signals in the presence of beta-cyclodextrin was observed.

Chapter 5

Results: ^{1}H-NMR studies on the interaction of racemic substrates with cyclodextrins

5.1 Introduction

5.1.1 ^{1}H-NMR spectra of cyclodextrins

The 270MHz ^{1}H-NMR spectrum of alpha-cyclodextrin in D_2O is shown in Figure 5.1a. The proton chemical shifts for the three common native cyclodextrins are listed in Table 5.1.

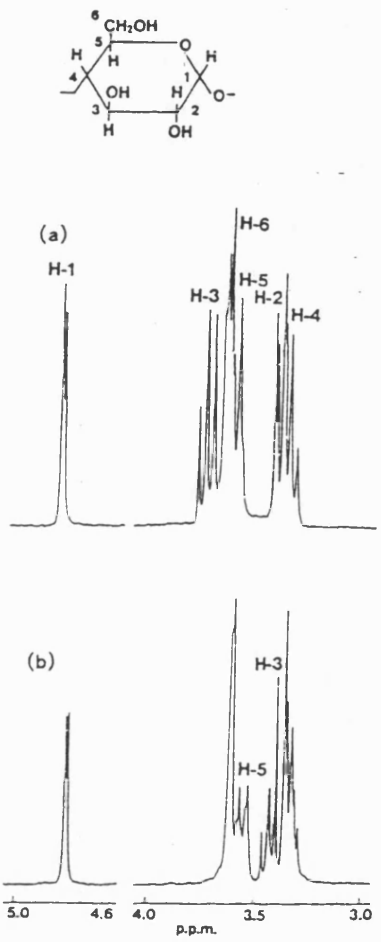
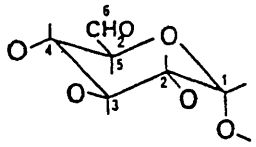


Figure 5.1. 270MHz ^{1}H-NMR spectra of (a) alpha-cyclodextrin (0.01M) and (b) alpha-cyclodextrin - para-nitrophenol (pNP) (0.05M) in phosphate buffer solution at pH 6.0. Reference (external): tetramethylsilane (0.00ppm). From Inoue (1987).

Assignments were made initially at 100MHz by Demarco and Thakkar (1970), and confirmed in subsequent studies at 220MHz (Wood *et al* (1977)) and 500MHz (Inoue *et al*

(1984)). All the glucose units in an underivatised cyclodextrin are equivalent, owing to the symmetrical nature of the molecule. This considerably simplifies the spectra. The spectra of the three native cyclodextrins are very similar, as Table 5.1. shows.

Table 5.1. 400MHz NMR characteristics of cyclodextrins in D_2O (from Casy and Mercer (1988). External reference: acetone (2.1ppm). Multiplet separations (Hz) in parentheses. Abbrevations: s = singlet; d = doublet; t = triplet; m = multiplet; b = broad; u = unresolved.



Cyclodextrin	H-1	H-2	H-3	H-4	H-5	6-CH_2
alpha-	5.03d	3.61dd	3.96t	3.59t	u	u
	(3.5)	(3.4, 10)	(9.5)	(9)		
beta-	5.05d	3.62dd	3.94t	3.56bt	u	u
	(4)	(3.5, 9.8)	(9.5)	(9)		
gamma-	5.08d	3.63dd	3.91t	3.59t	u	u
	(3.7)	(3.4, 9.8)	(9.5)	(9.5)		

Cyclodextrin conformations have been confirmed from coupling constants. These are consistent with conformations where each glucose unit is in the C1 chair conformation (Wood *et al* (1977)). In DMSO-d_6 solution, the hydroxyl proton resonances of cyclodextrins are observed. The temperature-dependance of the chemical shifts of these signals has

been used to confirm the presence of interglucosidic hydrogen bonds between C-2 and C-3 hydroxyls (St.-Jacques *et al* (1976)).

5.1.2. NMR spectra of cyclodextrin complexes

When a cyclodextrin complex is formed, changes may be induced in the NMR spectra of both the cyclodextrin and the substrate. This is exemplified in Figure 5.1b, which shows the changes induced in the ^{1}H-NMR spectrum of alpha-cyclodextrin on addition of para-nitrophenol (pNP) as guest. The changes shown in Figure 5.1b are typical of those caused by inclusion of an aromatic guest, *i.e.* shielding of the cyclodextrin cavity protons H-3 and H-5. This effect is due to the high pi-electron density above and below the plane of an aromatic ring. The changes in chemical shifts of the cyclodextrin protons on the outside of the cavity (H-1, H-2 and H-4) on complex formation are generally very small and less predictable.

Changes in substrate chemical shifts are also somewhat unpredictable. In the case of the alphaCD-pNP system, the substrate aromatic proton resonances move to lower field on complexation (Inoue *et al* (1984)). This is by no means always the case, however.

The complexation equilibrium is almost always fast on the NMR time-scale (Demarco and Thakkar (1970)). Thus, in most cases, only one signal is recorded for a given substrate proton, reflecting a weighted average of the signals due to the free and bound form of the substrate. Rapid spinning of the substrate within the cavity accounts for the NMR equivalence of the glucose residues of the cyclodextrin being preserved on addition of substrate. In recent reports, it has been shown that complexes with slow exchange on the NMR time-scale can be prepared (Saito *et al* (1990), Yoshida *et al* (1990)). Thus, in the NMR spectra of some polymethylene bis-(1-pyridinium) substrates with alpha-cyclodextrin, distinct NMR signals for the free and complexed forms of the substrate can be observed at

5°C, with coalesence occurring as temperature is increased. Line-broadening in the proton NMR spectrum of ß-cyclodextrin with anthraquinone sulphonate at low temperature has been attributed to "intermediate" exchange, although in this case full resolution of signals due to free and complexed forms was not seen (Djedaïni and Perly (1990)).

Spin-spin coupling constants also change on complex formation. Inoue *et al* (1981) have shown by analysis of coupling constants that the side-chain conformations of some aromatic amino acids are altered by complex formation with beta-cyclodextrin. Changes in coupling constants of underivatised cyclodextrins on complexation are generally small, consistent with the view that the cyclodextrin conformation is largely unaltered on inclusion of a guest (Wood *et al* (1977)).

5.1.3 Determination of complex stoichiometry from NMR data

The method of continuous variation, as originally described by Job (1928) has been widely used to determine the stoichiometry of cyclodextrin complexes. In this method, the total molar concentration of two complexing species is kept constant, and complex stoichiometry determined from the variation in some quantity reflecting the equilibrium mole fraction of complex with the initial mole fraction of one of the species.

In an NMR spectrum of a cyclodextrin CD complexed with a substrate S, assuming fast exchange on the NMR time-scale, observed cyclodextrin chemical shifts, δ_{obs}, are given by the weighted mean of the chemical shifts of the free (δ_{free}) and bound ($\delta_{compl.}$) cyclodextrin, *i.e.* $\delta_{obs} = ((\delta_{compl.}[S.CD_n]) + (\delta_{free}.([CD]-[S.CD_n])))/[CD]$ (eqn. 5.1), where $[S.CD_n]$ is the equilibrium concentration of the complex (of unknown stoichiometry), [CD] and [S] are the initial concentrations of cyclodextrin and substrate.

The change in chemical shift on addition of substrate to cyclodextrin, $\Delta\delta$, is given by $\Delta\delta = \delta_{obs} - \delta_{free}$, and hence

$$\Delta\delta .[CD] = (\delta_{compl.} - \delta_{free}).[S.CD_n] = \text{constant}. [S.CD_n]. \qquad \text{(eqn. 5.2)}$$

The equilibrium mole fraction of complex is therefore proportional to $\Delta\delta .[CD]/([S]+[CD])$.

The equilibrium constant for the complexation is given by

$$K_f=[S.CD_n]/([S_{free}].[CD_{free}]^n) \qquad \text{(eqn. 5.3)},$$

where $[S_{free}]$ and $[CD_{free}]$ are the equilibrium concentrations of free cyclodextrin and substrate. The total initial concentration of cyclodextrin and substrate is given by

$$[S]+[CD]=M. \qquad \text{(eqn. 5.4)}$$

The initial mole fraction of substrate, x, is given by

$$x = [S]/([CD]+[S]). \qquad \text{(eqn. 5.5).}$$

The concentration of free substrate and cyclodextrin are then given by

$$[S_{free}] = x.M - [S.CD_n] \qquad \text{(eqn. 5.6)}$$

and

$$[CD_{free}] = (1-x).M - n.[S.CD_n]. \qquad \text{(eqn. 5.7).}$$

The mole fraction of complex will be maximised when $d[S.CD]/dx = 0$. Differentiation of equations 5.3, 5.6, and 5.7 with respect to x, incorporating the above condition, gives

$$[CD_{free}].d[S_{free}]/dx + n.[S_{free}].d[CD_{free}]/dx = \mathbf{0} \qquad \text{(eqn. 5.8)},$$

$$d[S_{free}]/dx = M \qquad \text{(eqn. 5.9)}$$

and

$$d[CD_{free}]/dx = -M \qquad \text{(eqn. 5.10).}$$

Combination of equations 5.4, 5.5, 5.8, 5.9, and 5.10 yields the condition

$$x=1/(1+n) \qquad \text{(eqn.5.11)}$$

for maximisation of $[S.CD_n]$. Hence a plot of $\Delta\delta.[CD]/([S]+[CD])$ against x is maximised when equation 5.11 is satisfied. n may therefore be determined from such a plot.

Continuous variation plots have been used to determine the stoichiometry of complexes of ß-cyclodextrin with propanolol hydrochloride (Greatbanks and Pickford (1987)), dimethindene maleate (Mercer (1989)), anthraquinone sulphonate (Djedaïni and Perly (1990)), indomethacin (Djedaïni *et al* (1990a)), 5-fluorouracil and ftorafur (Wen and Cui (1990)) from NMR data. In all these cases, 1:1 complex stoichiometry was deduced, and complex

stability was high. A recent report has pointed out disadvantages of Job's method, most notably that the maximum in a Job plot may be poorly defined for weak complexes and that complications arise when competing equilibria occur, such as in a case where 2:1 complexes are also present (Gil and Oliveira (1990)).

5.1.4 Determination of complex formation constants

The variation of substrate chemical shifts with the mole ratio of substrate to cyclodextrin provides a useful method for the determination of cylodextrin complex stability constants. The procedure was originally described by Bergeron *et al* (1977a), and was a modification of that developed by Benesi and Hildebrand (1949).

For the equilibrium S + CD $\leftrightharpoons$ S.CD, the complex stability constant K_f is given by

$$K_f = [S.CD]/([S_{free}].[CD_{free}] = [S.CD]/(([S]-[S.CD])([CD]-[S.CD])) \quad \text{(eqn. 5.12).}$$

Assuming fast exchange on the NMR time-scale, the observed substrate chemical shift will be given by $\delta_{obsd} = ([S]-[S.CD]/[S]).\ \delta_{free} + ([S.CD]/[S]).\ \delta_{compl.}$ (eqn. 5.13).

The change in chemical shift, $\Delta\delta$, is given by

$$\Delta\delta = \delta_{obsd} - \delta_{free} \quad \text{(eqn. 5.14).}$$

Combining equations 5.13 and 5.14 gives, on rearrangement,

$$\Delta\delta = ([S.CD]/[S]).D \quad \text{(eqn. 5.15),}$$

where D is the limiting chemical shift change at infinite degree of complexation and is given by $D = \delta_{compl.} - \delta_{free}$ (eqn. 5.16).

Equation 5.12 may be rearranged to

$$[S.CD]/K_f = [S.CD]^2-[CD].[S.CD]-[S].[S.CD]+[CD].[S] \quad \text{(eqn.5.17).}$$

At this point, it is assumed that $[S.CD]^2 << [CD].[S]$, which is true for small degrees of complexation. With this approximation, and combined with equation 5.15, equation 5.17 becomes

$$[CD]/\Delta\delta = ([CD]+[S])/D + 1/(K_f.D) \qquad \text{(eqn. 5.18).}$$

The parameters D and K_f could therefore be determined from a linear plot of $[CD]/\Delta\delta$ vs. [CD]+[S].

Equation 5.18, and similar modifications of the Benesi-Hildebrand equation have been widely used for the determination of stability constants of cyclodextrin complexes (Vecchi *et al* (1988), Inoue *et al* (1984), Bergeron *et al* (1978), Inoue *et al* (1985), Djedaïni and Perly (1990), Djedaïni *et al* (1990a), Wen and Cui (1990)).

An alternative and more rigourous approach to the solution of the above equations has recently been provided by Wenz and von der Bey (1988) and by Smith *et al* (1989).

Equation 5.12 is transformed to

$$k=(x-y)(1-y)/y \qquad \text{(eqn. 5.19),}$$

where $y = [S.CD]/[S] = \Delta\delta/D$ (eqn. 5.20),

$x = [CD]/[S]$ (eqn. 5.21),

and $k = 1/(K_f.[S])$ (eqn. 5.22).

Rearrangement of equation 5.19 and solution of the resulting quadratic gives

$$y = (x+k+1)/2 - ((x+k+1)^2/4 - x)^{1/2} \qquad \text{(eqn. 5.23).}$$

Resubstitution of x, y and k then gives

$$\Delta\delta/D = ([CD]/[S] + 1/(K_f.[S]) + 1)/2 - (([CD]/[S] + 1/(K_f.[S]) + 1)^2/4 - [CD]/[S]))^{1/2}$$

(eqn. 5.24).

K_f and D are then obtained by non-linear regression analysis of the variation of $\Delta\delta$ with cyclodextrin concentration.

Wenz and von der Bey (1988) have compared results obtained by the two approaches. For the complex of perpentyl-ß-cyclodextrin with para-nitrophenol, use of equation 5.18 led to a K_f value of 1530 $dm^3\ mol^{-1}$ while use of equation 5.24 led to the value 3560 $dm^3\ mol^{-1}$. The significant difference between values obtained by the two methods was attributed to the the

approximation inherent in the Benesi-Hildebrand method that the mole fraction of complex present at equilibrium is small. Bergeron *et al* (1977a) also pointed out that, under certain experimental conditions, this approximation might give rise to significant error.

5.1.5 Determination of complex geometry and structure

A variety of NMR measurements have been used to give information concerning the structure and geometry of cyclodextrin complexes.

The magnitude of the proton chemical shift changes in cyclodextrin cavity protons on complex formation have been used to estimate the depth of penetration of aromatic guests into the cavity (Komiyama and Hirai (1981), Inoue *et al* (1984)). The shielding effect of an aromatic ring on a proton at a given position relative to the ring has been mapped by Johnson and Bovey (1958). From this theory, curves relating specifically to the inclusion of an aromatic guest in a cyclodextrin have been computed (Komiyama and Hirai (1980). The intrinsic chemical shifts of the cyclodextrin H-3 and H-5 protons in the complex, determined from equation 5.18 have been compared with the theoretical Johnson-Bovey curves, and an estimate made for the time-averaged position of the centre of the aromatic ring in the cyclodextrin cavity.

Changes in ^{13}C chemical shifts have also been used to estimate the position of a guest within the cavity (Gelb *et al* (1981), Inoue *et al* (1984, 1985a), Komiyama and Hirai (1981), Suzuki *et al* (1990)). The basis of these calculations is that changes in substrate ^{13}C chemical shifts are caused mainly by moving from solvent (water) into the relatively hydrophobic cyclodextrin cavity, and that the magnitude of these changes is directly related to the depth of cavity penetration. Inoue *et al* (1985a) have shown that this change is equivalent to moving from water into dioxan, and has carried out semi-empirical MO calculations using the dielectric constants of water and dioxan to predict the intrinsic

chemical shift change expected for a given cavity penetration.

Studies of the molecular dynamics of cyclodextrin-substrate systems have been used to gain structural information. The inclusion of a substrate in the cyclodextrin cavity causes a reduction in its molecular motion. This results in an increase in spin-lattice relaxation and hence a decrease in the spin-lattice relaxation time T_1. This effect has been widely reported from measurements of ^{13}C and ^{2}H T_1 values, and has been used to probe cyclodextrin-guest orientation (Uekama *et al* (1977b), Bergeron and Channing (1979), Behr and Lehn (1976), Smith and *et al* (1989), Suzuki *et al* (1990), Inoue *et al* (1987a)).

Nuclear Overhauser effects (nOe) have been demonstrated in cyclodextrin complexes. The nuclear Overhauser effect is a 'through-space' effect. Cyclodextrin-substrate intramolecular nOe's therefore indicate the proximity of cyclodextrin and substrate protons. This can give precise structural information. Bergeron and Rowan (1976) produced a 9% enhancement in the meta- protons signals of sodium 2,6-dimethyl-4-nitrophenolate by irradiation of the alpha-cyclodextrin H-3 cavity protons in a one-dimensional nOe difference experiment. From this, they were able to deduce that the substrate was entering the cyclodextrin cavity at the wide (2-,3- end) nitro- group first.

The results of a 2D-NOESY experiment on the alpha-cyclodextrin - pNP system were reported by Yamamoto *et al* (1987). Cross-peaks were observed connecting the cyclodextrin H-3 resonance to both ortho- and meta- substrate protons, while the cyclodextrin H-5 resonance connected only to the meta- substrate protons. From this information, a very clear picture of the complex structure in solution could be obtained.

Kitchin *et al* (reported at *2nd Int. Symp. Pharm. Biomed. Anal.*, York (1990)) used 2D-NOESY spectroscopy to investigate the structure of the ß-cyclodextrin-ibuprofen complex, and were able thereby to deduce the probable mode of inclusion. Greatbanks and

Pickford (1987) used 1D-nOe measurements to postulate the structure of the ß-cyclodextrin-propanolol complex.

Some authors, however, have reported difficulty in observing nOe effects in cyclodextrin complexes (Casy and Mercer (1988), Divakar (1990), Inoue *et al* (1989)). While this may in part be due to low complex stabilities and/or loose binding, there is a more fundamental reason why such experiments might fail, based on the way the magnitude of nuclear Overhauser effects depends on molecular weight (Neuhaus and Williamson (1989)).

The size of nOe's specifically depends on molecular tumbling rate, as described by the correlation time t_c. Small molecules, which tumble rapidly, give positive nOe's. Large molecules, which tumble slowly and thus have large t_c values, give negative nOe's. There is a cross-over point where nOe's are zero. This occurs when

$$\omega t_c \simeq 1.12 \qquad \text{(eqn. 5.25)},$$

where ω = Larmor frequency $\simeq$ spectrometer frequency x 2π and $t_c \simeq$ mol. wt. x 10^{-12} in typical solvents. Depending on solvent, temperature and spectrometer frequency, this condition is often met for molecules in the molecular-weight range 1000 to 2000. It is therefore not surprising that cyclodextrin complexes often have small or zero nOe's measured in the laboratory frame of reference.

An experiment overcoming this limitation, known as ROESY or CAMELSPIN, was first described by Bothner-By *et al* (1984). Its application to cyclodextrin complexes has recently been described (Inoue *et al* (1989)). In the ROESY experiment, nOe's are measured in a frame of reference rotating at the Larmor frequency. This is achieved by the application of a "spin-locking" field, generated by the spectrometer transmitter or decoupler during the nOe relaxation period or the NOESY mixing period. Viewed in this rotating frame, relative ω is close to zero and positive nOe's are obtained across the whole molecular weight range.

Inoue *et al* (1989) have demonstrated intramolecular cross-peaks in the 2D-ROESY spectrum of pNP with 6-O-(α-D-glucopyranosyl)- β-cyclodextrin, a system for which no nOe's could be observed in the laboratory frame. This data allowed them to postulate a structure for the complex.

5.1.6 Cyclodextrins as NMR chiral shift reagents

The changes in substrate chemical shifts caused by cyclodextrin complexation enable their use as NMR shift reagents. This was first demonstrated by MacNicol (1975), who used ß-cyclodextrin to simplify the proton spectrum of p-cumene. Much greater interest has been shown in the use of cyclodextrins as chiral shift reagents. This relies on the fact that the NMR spectra and/or stabilities of diastereomeric complexes formed between a pair of enantiomers and a cyclodextrin may be different. Thus, enantiotropic signals may be distinguished in the NMR spectrum of a racemate on addition of an appropriate cyclodextrin. This phenomenon was first reported by MacNicol and Rycroft (1977), who demonstrated enantiotropic splittings in the ^{19}F-NMR spectrum of racemic 1-phenyl-2,2,2-trifluoroethanol on addition of an excess of alpha-cyclodextrin.

Numerous subsequent reports of chiral discrimination induced in the NMR spectra of racemates by cyclodextrins have appeared. Dyllick-Brenzinger and Roberts (1980) reported chiral recognition induced in the ^{15}N spectrum of 8-benzyl-5,6,7,8-tetrahydroquinoline. Several 2-arylpropionic acids, including pirprofen (Uekama *et al* (1985)), ibuprofen (Casy and Mercer (1988)), and flurbiprofen (C.A. Marchant, Univ. of Bath, unpubl. data), have shown enantiotropic proton splittings in the presence of ß-cyclodextrin. A number of basic drugs have also shown chiral discrimination with cyclodextrins, including pheniramine antihistamines, analgesics such as alphaprodine and methadone (Casy and Mercer (1988)), and ß-blockers such as propanolol (Greatbanks and Pickford (1987)). Murakami *et al* (1988) investigated chiral recognition in mandelic acid, methyl mandelate, and

N-acetyl- α-phenylglycine. They found that considerably larger effects were induced by an acetylamino-derivatised ß-cyclodextrin than by the native compound. Certain amino acid derivatives have also shown chiral discrimination with cyclodextrins. (Smith and Spotswood (1989), Yamashoji *et al* (1990)).

The application of such phenomena to optical purity analysis has been discussed (Great-banks and Pickford (1987), Casy and Mercer (1988)). Whilst resolution of enantiotropic signals is not great enough in every case to detect low levels of an enantiomeric purity, trials have shown that optical impurities can be detected to the 1% level in several cases. The advantages of cyclodextrins as chiral shift reagents over other reagents (*e.g.* lanthanides, chiral solvents) are that experiments can be conducted in aqueous media (which can be important for water soluble chiral molecules), that cyclodextrins do not induce line-broadening in substrate spectra and that cyclodextrin proton resonances are in a relatively favourable region of the spectrum (*i.e.* they do not obscure most substrate resonances).

5.2 Tetrahydroisoquinolines

5.2.1 ^{1}H-NMR spectrum of TQ1

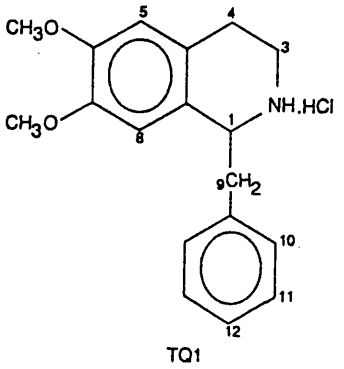
Chemical shifts of proton signals of TQ1 were assigned as shown in table 5.2. Assignments were made with the aid of an nOe experiment. Irradiation of the aromatic proton singlet at 6.5ppm caused clear enhancement of the C-H triplet at 4.8ppm (2.9%), the methoxyl singlet at 3.6ppm (2.7%) and the benzylic methylene signal at 3.4ppm (1.8%), allowing assignments to be made as shown. The H-8 proton was observed to be highly shielded, presumably due to its proximity to the phenyl ring in the preferred conformation.

5.2.2 Effect of cyclodextrins on ^{1}H-NMR spectrum of ±-TQ1

Changes in chemical shifts of TQ1 protons were observed on addition of a mole equivalent of ß-cyclodextrin, as shown in table 5.2, and illustrated in figure 5.2. The most noticeable effect was an upfield shift in the H-8 signal, accompanied by significant chiral discrimination. This increased shielding was thought to be indicative of a conformational change in the substrate on complexation, with the H-8 proton moving nearer the region of high electron density around the phenyl ring. The cavity protons (H-3) and (H-5) of ß-cyclodextrin were shielded on interaction with TQ1, indicating inclusion of an aromatic moiety.

Interaction with TQ1 with a number of other cyclodextrins was investigated. The results are summarised in table 5.2. In all cases, some chiral discrimination in the H-8 signal was observed. The stereospecific interaction of a solute with all three underivatised cyclodextrins is previously unreported. It may be that the tetrahydroisoquinoline moiety includes in the larger host cavities, with the phenyl group including in smaller cyclodextrins, although there is no direct evidence to support this hypothesis. The largest changes in chemical shift, and most significant chiral discrimination, were observed with methyl-ß-cyclodextrin.

Table 5.2. ^{1}H-NMR chemical shifts of ±-TQ1 (a) alone (b) on addition of cyclodextrin (one mole equivalent). In cases where chiral discrimination is observed, chemical shifts of signals due to both enantiomers are noted. Abbreviations as Table 5.1, except n.r. = not resolved from other signals; HP-betaCD = hydroxypropyl-ß-cyclodextrin (MS = 0.6); Me-betaCD = methyl-ß-cyclodextrin (DS = 1.8).



TQ1

proton(s)	chemical shifts, ppm					
	no CD	+ alphaCD	+ betaCD	+ gammaCD	+ HP-betaCD	+Me-betaCD
H-1	4.79 (t)	n.r.	n.r.	4.79	n.r.	n.r.
3-CH_2	3.44 (m)	3.44	3.38	3.46	3.30	n.r.
4-CH_2	3.12 (m)	3.12	3.14	3.12	3.10	n.r.
9-CH_2	3.58 (ddd)	3.3	3.50	3.28	3.51	n.r.
H-5	6.92(s)	6.95	6.93	6.93	6.91	6.904, 6.896
H-8	6.57(s)	6.50, 6.60	6.40, 6.32	6.40, 6.35	6.41, 6.39	6.19, 6.00
H-10	7.30 (dd)	7.32	7.28	7.31	n.r.	n.r.
H-11	7.4 (m)	7.4	7.4	7.4	n.r.	n.r.
H-12						
CH_3-O	3.85 (s)	3.90	3.87	3.88	3.84	3.90
	3.67 (s)	3.71	3.62	3.66	3.62	n.r.

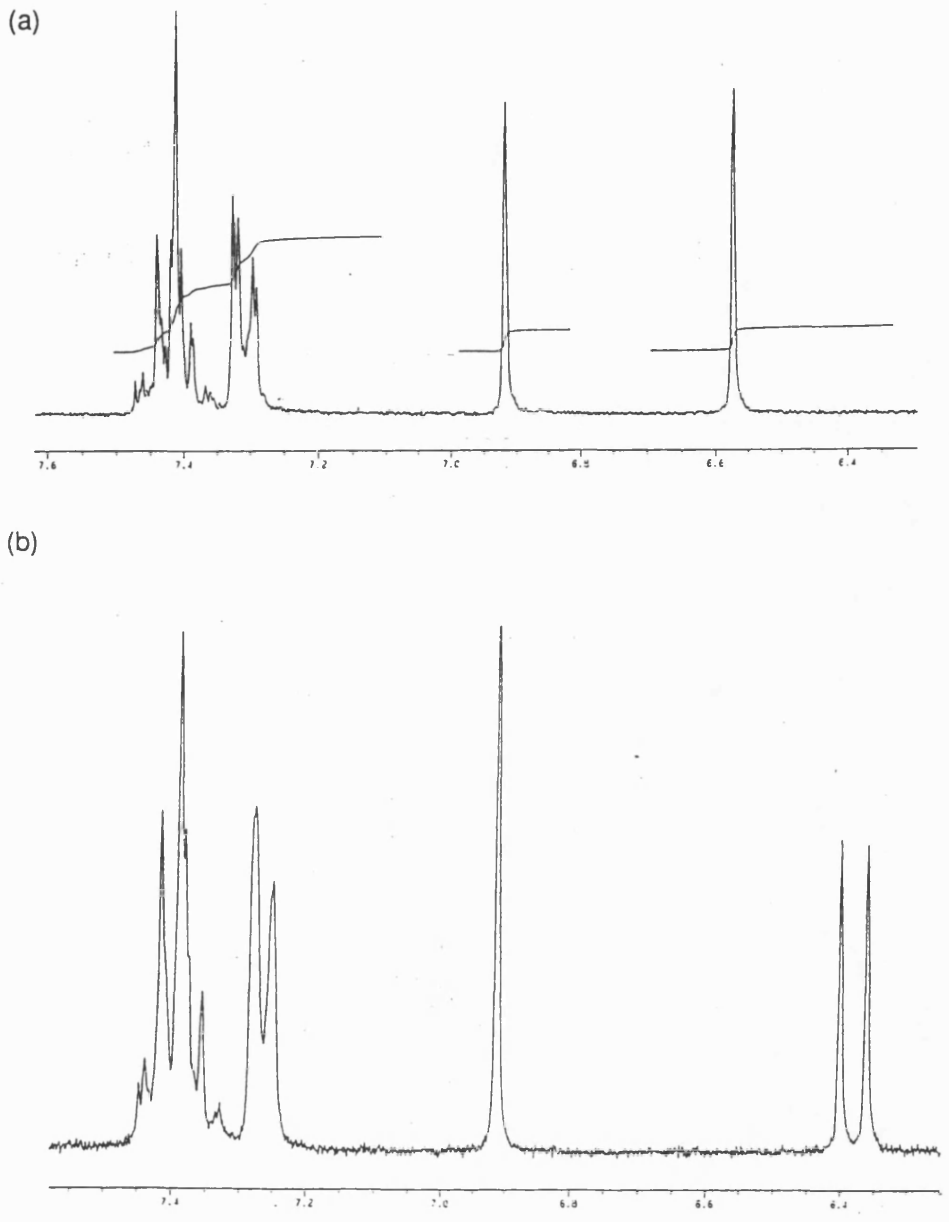


Figure 5.2. Aromatic proton regions of 270MHz ^{1}H-NMR spectra of ±-TQ1 in D_2O (a) alone (b) in the presence of ß-cyclodextrin (one mole equivalent).

Near-baseline (>90%) resolution of enantiotropic signals at 270MHz was induced by addition of betaCD, gammaCD and Me-betaCD to TQ1, with greatest resolution in the latter case. The optical purity of partially resolved samples of this compound might therefore be determined by NMR using cyclodextrins as chiral shift reagents.

5.2.3 Effect of ß-cyclodextrin on ^{1}H-NMR spectra of other tetrahydroisoquinolines.

The changes induced in NMR spectra on addition of a mole equivalent of ß-cyclodextrin to racemic solutes in this series are summarised in Table 5.3. Five of the seven compounds exhibited chiral discrimination with ß-cyclodextrin, although not to such a large degree as TQ1. In contrast to TQ1, de-shielding of the H-8 protons on complexation was observed in several cases. Thus, the conformational changes induced by complexation in this series differ from compound to compound. In the case of TQ4, slight chiral discrimination was also observed in the H-5 aromatic signal. TQ3 and TQ7 showed no chiral discrimination, and there was no evidence for inclusion either through changes in cyclodextrin or substrate signals. This suggests that the presence of methoxyl groups on the phenyl ring hinders complexation.

Table 5.3. Chemical shifts of H-8 protons of tetrahydroisoquinolines (a) alone (b) on addition of one mole equivalent of ß-cyclodextrin. In cases where chiral discrimination is observed, chemical shifts of signals due to both enantiomers are noted.

substrate	H-8 chemical shifts, ppm	
	no CD	+ betaCD
TQ1	6.57	6.40, 6.32
TQ2	6.49	6.540, 6.545
TQ3	6.49	6.50
TQ4	6.52	6.540, 6.547
TQ5	6.52	6.440, 6.458
TQ6	6.40	6.390, 6.403
TQ7	6.75	6.78

Only in one case (TQ5) was near-baseline resolution of enantiotropic signals observed under the conditions employed. In this case, the experiment performed here might form the basis of a method of optical purity determination.

The effect of ß-cyclodextrin on 1,2,3,4-tetrahydroisoquinoline itself was investigated in an analogous NMR experiment. Shielding of cyclodextrin cavity protons was observed, indicating that inclusion was occurring. Thus, the presence of a phenyl substituent on the tetrahydroisoquinoline is not necessary for complexation, although there is evidence above that such a substituent affects the complexation of the solutes studied here.

5.2.3 Formation constant determination for the ß-CD:±-TQ1 system

The K_f values for the complexation of TQ1 enantiomers with ß-cyclodextrin were determined from the variation of H-8 chemical shifts with cyclodextrin concentration, as illustrated in figure 5.3. The K_f values and NMR parameters determined are given in Table 5.4.

The formation constants were fairly low by comparison with reported values for some other substrates. The K_f values for the two enantiomers were not significantly different (*i.e.* the standard error of the difference between them was greater than the difference, as shown in table 5.4). The difference in chemical shifts of the two diastereomeric complexes was shown to have greater statistical significance, and is therefore more likely to account for the observed enantioselectivity.

Table 5.4 Stability and ^{1}H-NMR chemical shifts of the complexes formed by TQ1 enantiomers with ß-cyclodextrin in D_2O at pH 5. Values obtained from data in Figure 5.3 according to Appendix 1 using MINSQ.

parameter		value	std.dev.
complex formation constant, M^{-1},	K_{f1}	63.1	6.5
	K_{f2}	66.5	5.5
chemical shift change on complexation, ppm	D_1	-0.591	0.039
	D_2	-0.699	0.036
difference in stability constants, M^{-1}	K_{f2}-K_{f1}	3.5	8.6
difference in complex chemical shifts, ppm	D_1-D_2	0.107	0.053
correlation coefficient,	r	0.9995	

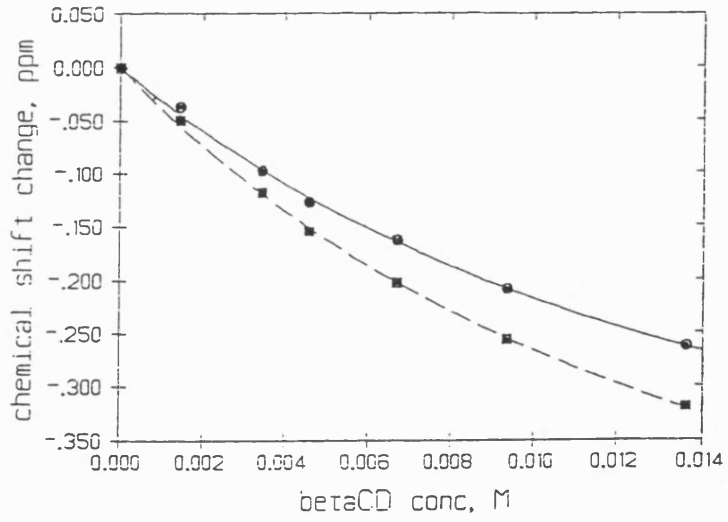


Figure 5.3. Variation in chemical shifts of H-8 protons of TQ1 enantiomers with ß-cyclodextrin concentration. TQ1 racemate concentration = 2.026 mM in 0.1M phosphate buffered D_2O at pH 5. Curves fitted to the equations A.16 and A.17 (appendix 1) by non-linear least-squares regression using MINSQ.

5.3 Phenothiazines

5.3.1 Chiral discrimination induced by ß-cyclodextrin and derivatives

ß-cyclodextrin was found to induce chiral discrimination in the proton NMR spectra of a number of chiral phenothiazines. The most marked effects were observed for trimeprazine, as illustrated in Table 5.5, where ß-cyclodextrin caused enantiotropic splitting of the dimethylamino (2.8ppm) and C-methyl (1.1ppm) signals. In the former case, the enantiotropic signals were baseline resolved at 270MHz. This experiment would therefore provide the basis for a sensitive optical purity determination method for this material.

Interpretation of the spectra was complicated by non-equivalence of prochiral N-methyl groups. Thus in the spectra of the achiral solute prothipendyl, duplication of the dimethylamino signal was observed on addition of ß-cyclodextrin. In the spectra of trimeprazine tartrate (Table 5.5), the two N-methyl groups were equivalent in the absence of ß-cyclodextrin, but became non-equivalent in the presence of ß-cyclodextrin. Only one of the N-methyl signals exhibited enantiotropic splitting at 270MHz, giving three signals in all. In the spectra of the trifluoroacetate salt (Figure 5.4), the N-methyl groups were non-equivalent in the absence of ß-cyclodextrin (due perhaps to the formation of a tight ion-pair with greater conformational rigidity than the tartrate). On addition of ß-cyclodextrin, both N-methyl signals were duplicated (although only one was well resolved), giving four signals in all.

On addition of ß-cyclodextrin to a resolved sample of one of the enantiomers, produced by the method described in chapter 4, only two major N-methyl signals were observed, with some evidence of optical impurity (Figure 5.4d).

Table 5.5. NMR chemical shifts of methyl protons of racemic phenothiazine salts in D_2O (a) alone (b) on addition of one mole equivalent of cyclodextrin. Where splitting of enantiotropic and/or prochiral methyl signals occurred, the chemical shift of each observed signal is given.

substrate	cyclodextrin	$\delta_{N\text{-}Me}$, ppm		$\delta_{C\text{-}Me}$, ppm	
		no CD	+ CD	no CD	+ CD
trimeprazine tartrate	betaCD	2.42	2.75, 2.62, 2.53	0.86	1.16, 1.19
	Me-betaCD		2.69		1.18, 1.20
promethazine.HCl	betaCD	2.68	2.80, 2.82	1.33	1.47, 1.49
	Me-betaCD		2.792, 2.800		1.454. 1.468
	HP-betaCD		2.805, 2.816		1.462, 1.477
dimethothiazine mesylate	betaCD	2.59	2.794, 2.800 2.72	1.31	1.432, 1.440
isothipendyl.HCl	betaCD	2.76	2.837, 2.852	1.36	1.47, 1.48
	Me-betaCD		2.837, 2.844		1.468, 1.472
methotrimeprazine*	betaCD	2.71	2.89, 2.77, 2.69	1.17	1.31, 1.34

* = 1:1 mixture of (-)-methotrimeprazine maleate and (+)-methotrimeprazine tartrate.

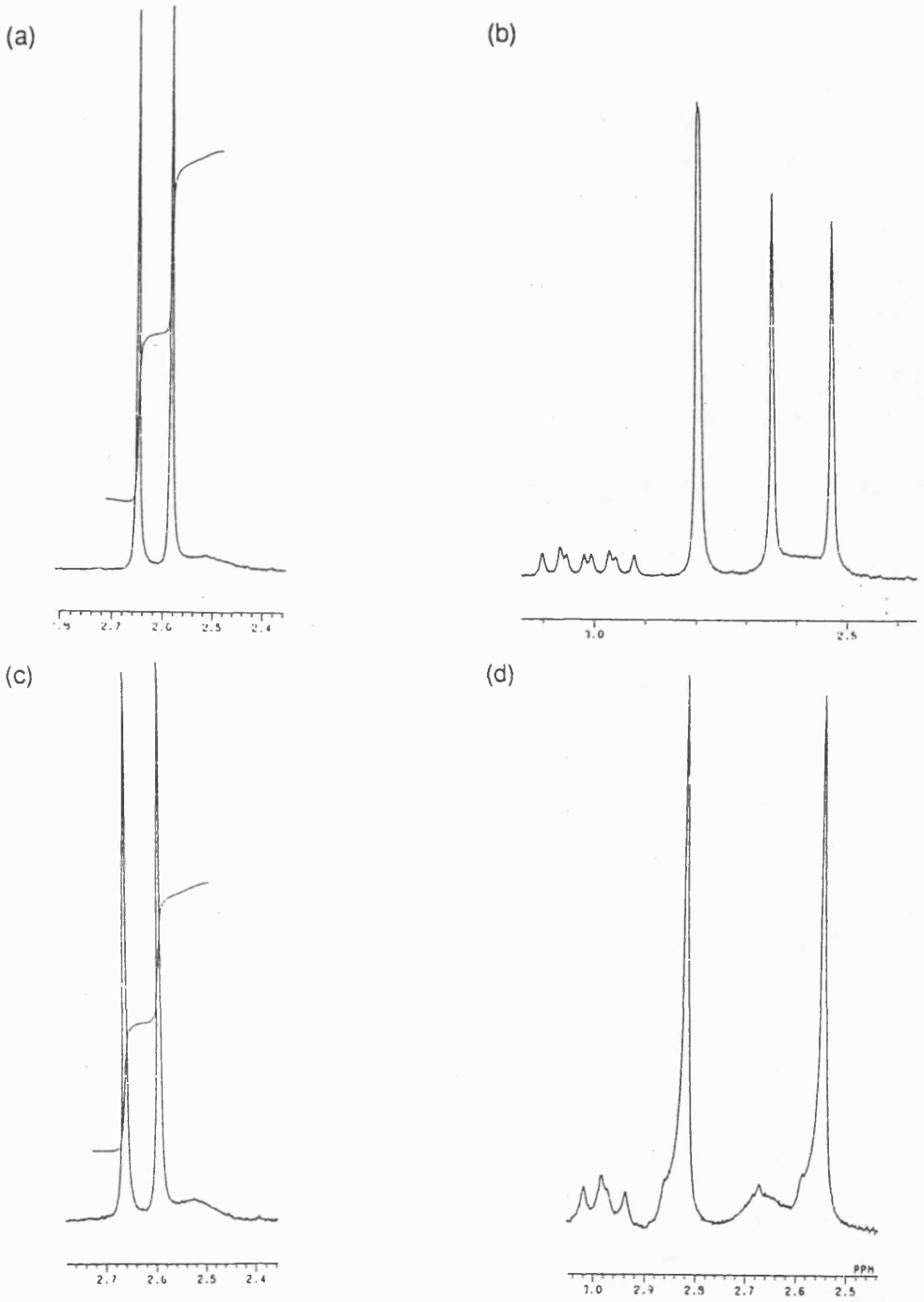


Figure 5.4. High-field regions of 270MHz spectra of trimeprazine trifluoroacetate in D_2O (a) racemate alone: (b) racemate in the presence of one mole equivalent of ß-cyclodextrin: (c) recovered HPLC peak 1 after semi-prep resolution: (d) HPLC peak 1 in the presence of one mole equivalent of ß-cyclodextrin.

Table 5.5 also summarises the data obtained for the other phenothiazines studied. In all five cases, chiral discrimination was evidenced by duplication of alkyl methyl signals. Duplication or triplication of dimethylamino signals was also observed in all cases. Where duplication of the latter occurred, it was not clear whether this was due to enantiotropic splitting or induced N-methyl non-equivalence. Pure samples of the enantiomers of these compounds would therefore be required to validate any chiral assay based on this approach.

5.3.2. Variation of ±-trimeprazine tartrate chemical shifts with ß-cyclodextrin concentration.

The effect of ß-cyclodextrin concentration on the chemical shifts of enantiotropic trimeprazine N-methyl proton signals is illustrated in Figure 5.5.

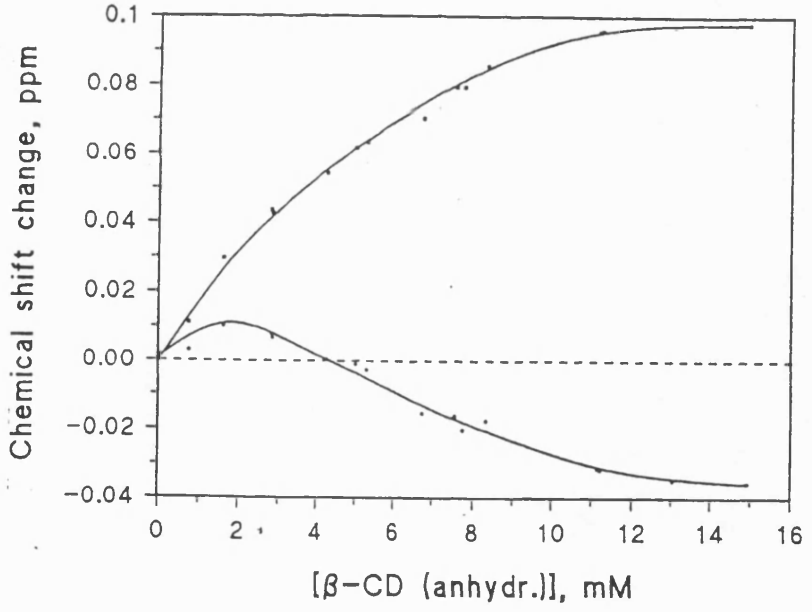


Figure 5.5. Variation of the dimethylamino proton chemical shifts of trimeprazine enantiomers in D_2O with ß-cyclodextrin concentration. [±-trimeprazine hemi-(+)-tartrate] = 10.65mM.

It seems unlikely from the shape of the curves obtained that the complexes formed have the same structure and/or composition across the cyclodextrin concentration range. This is particularly apparent in the lower curve, where the chemical shift change on complexation is positive at low cyclodextrin concentrations but negative at high cyclodextrin concentrations. Calculation of K_f for the complexes of trimeprazine enantiomers with ß-cyclodextrin was not attempted, as the model (appendix 1) assumes constant 1:1 complex stoichiometry across the concentration range.

Otagiri *et al* (1976) postulated that dimerisation of ß-cyclodextrin-phenothiazine complexes may occur, due to hydrogen bond formation between the amino group of one complex and the cyclodextrin hydroxyls of another. This may account for the observed behaviour, both the induced N-methyl non-equivalence in the presence of ß-cyclodextrin and deviations from the expected varation of chemical shifts with cyclodextrin concentration. The latter may occur due to changes in the extent of dimerisation across the cyclodextrin concentration range.

5.4 Mandelic acids and related compounds

5.4.1 Chiral discrimination induced by cyclodextrins

A number of aromatic acids and esters exhibited chiral discrimination with cyclodextrins, as shown in Table 5.6. Of the 21 compounds studied in this group, 18 caused shielding of ß-cyclodextrin cavity protons, indicating complex formation. Of these, 13 showed chiral discrimination induced by ß-cyclodextrin. In some cases, particularly monosubstituted acids, this took the form of duplication of the signal for the methine proton at the chiral centre. Where the aromatic ring was disubstituted, chiral discrimination in aromatic proton signals was frequently observed, particularly in signals due to protons meta- to the chiral substituent. In the cases of the mandelate esters studied, duplication of ester alkyl signals was observed.

Table 5.6. Summary of results of 270MHz ^{1}H-NMR experiments involving the addition of a mole equivalent of cyclodextrins to mandelic acids and related compounds in D_2O. Upfield shifts in cyclodextrin cavity proton signals taken to indicate complex formation. Substrate signal duplication taken to indicate chiral discrimination.

compound	cyclodextrin	evidence of complexation	chiral discrimination
mandelic acid	betaCD	yes	yes
	alphaCD	no	no
	methyl-betaCD	yes	no
" " sodium salt	betaCD	yes	no
tropic acid	betaCD	yes	no
	alphaCD	yes	no
3-phenyl-lactic acid	betaCD	yes	yes
	alphaCD	no	no
" " " sodium salt	betaCD	yes	yes
2-phenyl propionic acid	betaCD	yes	yes
	hydroxypropyl-betaCD	yes	yes
	methyl-ß-CD	yes	yes
" " " sodium salt	betaCD	yes	no
2-methoxy phenylacetic acid	betaCD	yes	no
	alphaCD	yes	no
	methyl-ß-CD	yes	no

Table 5.6 (contd.)

compound	cyclodextrin	evidence of complexation	chiral discrimination
O-acetyl-mandelic acid	betaCD	yes	yes
	alphaCD	yes	no
	hydroxypropyl-betaCD	yes	yes
	methyl-betaCD	yes	yes
" " " sodium salt	betaCD	yes	no
2-chloro-mandelic acid	betaCD	yes	no
	methyl-betaCD	yes	yes
4-chloro-mandelic acid	betaCD	yes	no
3-hydroxy-mandelic acid	betaCD	yes	yes
4-hydroxy-mandelic acid	betaCD	yes	yes
" " " sodium salt	betaCD	yes	no
2-methoxy-mandelic acid	betaCD	yes	no
3-methoxy-mandelic acid	betaCD	yes	yes
" " " sodium salt	betaCD	yes	no
4-methoxy-mandelic acid	betaCD	yes	yes
	hydroxypropyl-betaCD	yes	yes
cyclohexylphenylacetic acid (Na salt)	betaCD	yes	yes
phenylalanine	betaCD	no	no
3,4-dihydroxyphenylalanine	betaCD	no	no

Table 5.6 (contd.)

compound	cyclodextrin	evidence of complexation	chiral discrimination
tryptophan	betaCD	yes	yes
aminophenylacetic acid	betaCD	no	no
methyl mandelate	betaCD	yes	yes
ethyl mandelate	betaCD	yes	yes
benzoin	betaCD	yes	yes

In general, complexation of sodium salts of acids in this group was not strong, *i.e.* shifts in cyclodextrin cavity protons on addition of substrate were small. Chiral discrimination was absent in all of these cases except 3-phenyllactic acid sodium salt

Of the five compounds tested with alpha-cyclodextrin, only three showed any evidence for complex formation, and even in these cases no chiral discrimination was observed. This result is surprising considering the small size of the substrates studied in this group, which might be thought to be ideal for inclusion into the smaller alpha-cyclodextrin cavity.

Three of the compounds were tested with hydroxypropyl-ß-cyclodextrin (DS = 0.6). Similar results to those seen with unsubstituted ß-cyclodextrin were obtained.

Five of the racemates were tested with methyl-ß-cyclodextrin (DS = 1.8). In one case (2-chloromandelic acid), chiral discrimination was observed where none had been obtained with ß-cyclodextrin itself. Some qualitative and quantitative differences were observed in other cases, possibly indicating different modes of complexation for the two cyclodextrins under comparison.

Near-baseline resolution of enantiotropic signals under the conditions employed was only observed in a few cases. The best case was O-acetylmandelic acid with ß-cyclodextrin, where more than 90% resolution of the acetyl methyl singlet was observed. In several cases, complex signal multiplicities hindered resolution. In other cases, the degree of enantiotropic splitting was not large enough for baseline resolution to be observed even in singlet signals.

5.4.2 Formation constant determination for the methyl mandelate:ß-cyclodextrin system.

The formation constants for the complexes of R-(-)-methyl mandelate and S-(+)-methyl mandelate with ß-cyclodextrin were calculated from the experimental variation of methyl mandelate chemical shifts with cyclodextrin concentration. The data are illustrated in Figures 5.7. The results obtained are shown in tables 5.7 and 5.8.

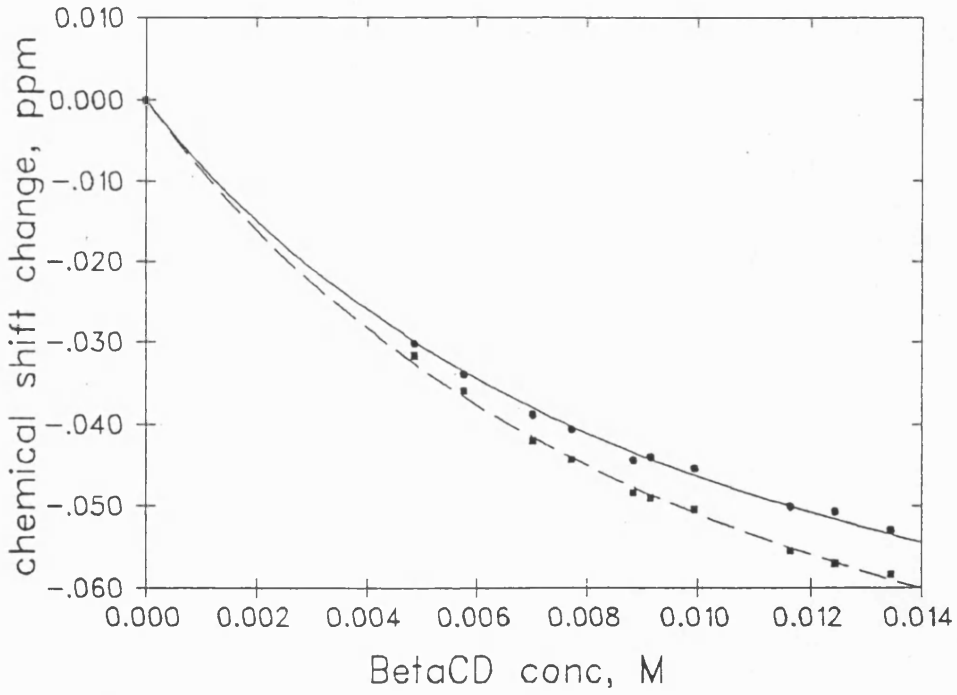


Figure 5.6 Variation in methine proton chemical shifts of the enantiomers of methyl mandelate in D_2O with ß-cyclodextrin concentration. [±-methyl mandelate] = 1.11mM. Curves fitted to equations A.16 and A.17 (appendix 1) using MINSQ.

Table 5.7. Stability and chemical shift data for the complexation of methyl mandelate enantiomers with ß-cyclodextrin, determined from separate NMR measurements on the individual enantiomers. The data was treated according to Smith *et al* (1989), and parameters were determined using MINSQ.

substrate	parameter		value	std. dev.
R(-)-MM	complex formation constant, M^{-1},	K_{fR}	91.1	3.9
	methine shift change on complexation, ppm	$D_{(CH),R}$	-0.1098	0.0026
	ester Me shift change on complexation, ppm	$D_{(CH3),R}$	0.0355	0.0010
	correlation coefficient	r	0.9999	
S(+)-MM	complex formation constant, M^{-1}	K_{fS}	89.6	5.1
	methine shift change on complexation, ppm	$D_{(CH),S}$	-0.0961	0.0030
	ester Me shift change on complexation, ppm	$D_{(CH3),S}$	0.0456	0.0015
	correlation coefficient	r	0.9998	
difference	stability constants, M^{-1}	K_{fR}-K_{fS}	1.6	6.5
	methine complex shifts, ppm,	$D_{(CH),S}$ - $D_{(CH),R}$	0.0137	0.0040
	ester Me complex shifts,	$D_{(CH3),S}$ - $D_{(CH3),R}$	0.0100	0.0018

Table 5.8. Stability and chemical shift data for the complexation of methyl mandelate enantiomers with ß-cyclodextrin, determined simultaneously according to appendix 1 from measurements on the racemate. Non-linear regression analysis was carried out using MINSQ.

parameter		value	std. dev.
complex formation constant, M^{-1}	K_{f1}	104.4	7.0
	K_{f2}	99.6	6.1
methine shift change on complexation, ppm	D_1	-0.0934	0.0032
	D_2	-0.1051	0.0033
difference in stability constants, M^{-1}	$K_{f2}-K_{f1}$	4.8	9.3
difference in complex methine shifts, ppm	D_1-D_2	0.0117	0.0046
correlation coefficient	r	0.9993	

Table 5.9. Statistical comparison of parameters for methyl mandelate-ß-cyclodextrin complexes determined from measurememts on the individual enantiomers (Table 5.7) or on the racemate (Table 5.8).

comparison		difference	std. dev.
formation constants,	$K_{f1}-K_{fS}$	14.8	8.7
	$K_{f2}-K_{fR}$	8.5	7.2
complex chemical shifts	$D_2-D_{(CH),R}$	0.0047	0.0042
	$D_1-D_{(CH),S}$	0.0027	0.0044

Table 5.9 illustrates that the parameters deduced from experimental measurements on the racemate were not significantly different from those obtained from experiments on the individual enantiomers. This indicates that the use of racemate data may be a valid approach, even though the equations are not explicitly soluble for such a system. Greater precision, however was obtained in experiments on the individual enantiomers (Table 5.7). More definite conclusions could therefore be drawn from these data sets.

The difference in stabilities (*i.e.* K_f values) of the complexes of the two enantiomers was not statistically significant (*i.e.* the difference was smaller than the standard deviation of the difference), but the chemical shifts of the complexes were seen to be significantly different Thus, the differences in complex shifts were 3 to 5 times the standard errors of those quantities. The observed enantioselectivity was therefore thought to arise largely from this factor, *i.e.* differences in D values (equation 5.24), rather than from differences in the K_f values.

5.5 Thromboxane antagonists

5.5.1 Assignment of proton NMR spectrum of ±-TA1

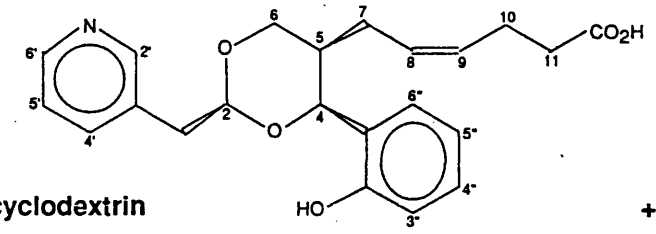
Assignment of signals in the 400MHz ^1H spectrum of TA1 were made as shown in Table 5.10. Aromatic (phenol and pyridyl) signals were well resolved and assigned on the basis of their chemical shifts and multiplicities. Other assignments were made using spin decoupling experiments, and from a ^1H-^1H COSY spectrum of ±-TA1 in CD_3OD. These demonstrated the existence of spin-spin coupling between CH_2-pyr and dioxan H-2; between dioxan H-4 and H-5; between H-5 and H-4, 6-CH_2 and side-chain 7-CH_2; between alkene H-8 and H-9 and 7-CH_2; between alkene H-9 and 10-CH_2; and between 10-CH_2 and 11-CH_2. The *cis*- (1,2 axial-equatorial) relationship of substituents at positions 4 and 5 was confirmed by measurement of the small (3Hz) coupling between H-4 and H-5.

A ^1H-nOe experiment was conducted in order to ascertain the stereochemical relationship between dioxan ring protons H-2 and H-4. Irradiation of the H-2 signal caused an enhancement of the H-4 signal, confirming their close proximity, which can only be explained in terms of a *cis*- (1,3-diaxial) relationship.

5.5.2 Chiral discrimination induced in the ^1H-NMR spectrum of ±TA1 by ß-cyclodextrin

The effect of the addition of a mole equivalent of ß-cyclodextrin on the ^1H-NMR spectrum of ±-TA1 sodium salt is illustrated in Figure 5.7. The most significant effects were observed in the aromatic region of the spectrum, which was clearly resolved. Duplication of all four pyridyl proton signals and one of the phenol signals was observed. Some changes were also observed in the alkyl chain signals (1.5 - 2.5ppm region), but these signals were not clearly resolved. Resolution of enantiotropic signals was most marked for the pyridyl H-2' signal, which might usefully be applied to optical purity determination for partially resolved samples of this compound. Changes were also induced in the cyclodextrin proton signals.

Table 5.10. Effect of addition of ß-cyclodextrin (one mole equivalent) on the 400MHz ^{1}H-NMR spectrum of ±TA1 (sodium salt) in D_2O. Internal ref.: HDO (4.90 ppm).



chemical shift, ppm	no cyclodextrin multiplicity	integral	assignment	+betaCD (1:1) chemical shift, ppm
8.61	d	1	pyridyl H-2'	8.71, 8.67
8.52	dd	1	pyridyl H-6'	8.57, 8.55
7.98	dt	1	pyridyl H-4'	7.96, 7.94
7.53	dd	1	pyridyl H-5'	7.60, 7.59
7.35	dd	1	phenol H-6"	7.36, 7.33
7.30	td	1	phenol H-4"	7.30
7.07	td	1	phenol H-5"	6.95 (n.r.)
6.95	dd	1	phenol H-3"	6.95 (n.r.)
5.40	m	1	alkene H-9	5.4 (n.r.)
5.20	m	1	alkene H-8	5.4 (n.r.)
5.40	d	1	dioxan H-4	5.4 (n.r.)
5.32	dd	1	dioxan H-2	5.4 (n.r.)
4.10	dd	2	dioxan 6-CH_2	4.1 (n.r.)
1.9	m	3	dioxan H-5, side-chain 10-CH_2	2.0 (n.r.)
3.19	m	2	CH_2-pyr	3.28
2.1	m	3	7-CH_{2a}, 11-CH_2	2.1 (n.r.)
1.6	m	1	7-CH_{2b}	1.6 (n.r.)

In particular, the H-5 signal was shifted markedly upfield, indicating inclusion of an aromatic guest.

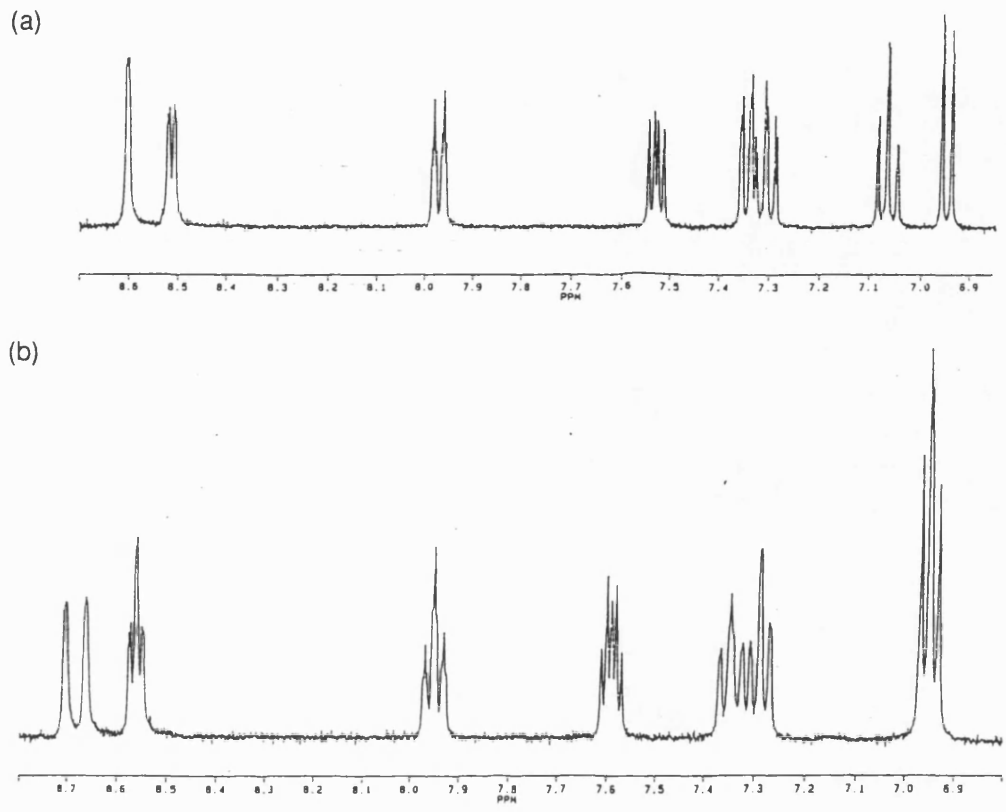


Figure 5.7. Aromatic proton regions of 400MHz ^{1}H-NMR spectra of ±-TA1 (sodium salt) in D_2O (a) alone (b) on addition of ß-cyclodextrin (one mole equivalent).

5.5.3 NMR chiral discrimination in other thromboxane antagonists

In all, seventeen thromboxane antagonist sodium salts were tested for chiral discrimination in NMR using ß-cyclodextrin. The results obtained are summarised in Table 5.11. All seventeen compounds caused shielding of cyclodextrin cavity protons, indicating that complexation was occurring. All but four compounds exhibited chiral discrimination. Enantiotropic splittings were most commonly observed in pyridyl signals, perhaps reflecting the well resolved nature of these signals in the spectrum of the uncomplexed solute.

Table 5.11. Summary of results of 270MHz ^{1}H-NMR experiments involving the addition of one mole equivalent of ß-cyclodextrin to thromboxane antagonist sodium salts in D_2O.

compound	evidence of inclusion	chiral discrimination in pyridyl signals	chiral discrimination in aryl signals	chiral discrimination in other signals
TA1	yes	yes	yes	no
TA2	yes	yes	yes	yes
TA3	yes	yes	no	yes
TA4	yes	no	no	yes
TA5	yes	no	no	no
TA6	yes	yes	no	yes
TA7	yes		no	yes
TA8	yes		no	yes
TA9	yes		no	yes
TA10	yes		yes	yes
TA11	yes		no	no
TA12	yes		no	no
TA13	yes	yes	no	yes
TA14	yes	yes	no	no
TA15	yes	yes	no	yes
TA16	yes	no	no	no
TA17	yes	yes	no	no

Other signals, notably those due to phenolic protons, dioxan ring protons and side-chain (R) protons exhibited chiral discrimination. Poor resolution, particularly of signals with high multiplicity, may have hindered observation of chiral discrimination in some cases. Thus, even in cases where no enantiotropic splittings were observed, it could not be categorically stated that the stabilities or chemical shifts of the diastereomeric complexes were identical.

Baseline resolution of enantiotropic signals was observed in six cases under the conditions employed. In these cases at least, this method might be used as the basis for chiral analysis.

5.5.4 NMR evidence for inclusion of model compounds.

2-isopropylphenol and 3-ethylpyridine were chosen as model compounds to mimic structural features of some of the thromboxane antagonists studied above. On addition to ß-cyclodextrin in 1:1 mole ratio, significant upfield shifts in cyclodextrin cavity proton signals were observed in both cases. It was thus demonstrated that both aromatic moieties in compounds such as TA1 might be capable of inclusion in ß-cyclodextrin.

5.5.5 Job (Continuous Variation) Plot for the ±-TA1-ß-cyclodextrin system

The stoichiometry of complexation between ±-TA1 (sodium salt) and ß-cyclodextrin was determined by Job's method. Changes in cyclodextrin proton signals were used for this plot, as these were larger than the changes in substrate chemical shifts. The resulting continuous variation plots are shown in Figure 5.8. All three curves showed a y maximum close to x=0.5, which suggested that the stoichiometry of the complex formed between TA1 and ß-cyclodextrin was 1:1.

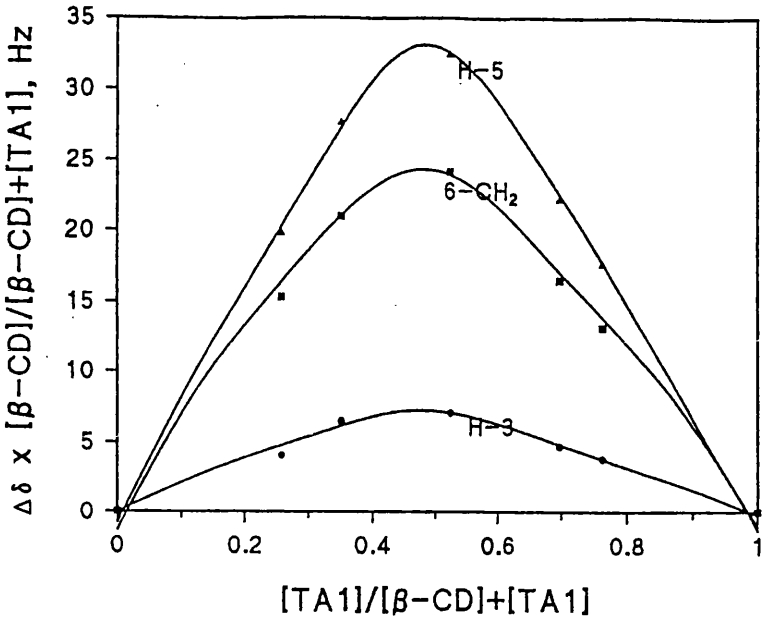


Figure 5.8. Job (continuous variation) plot for changes in cyclodextrin chemical shifts with ±TA1:ß-cyclodextrin ratio.

5.5.6 Determination of the formation constant for the ±-TA1-ß-cyclodextrin constant.

The formation constant for the ±-TA1-ß-cyclodextrin 1:1 complex was determined from the variation of the pyridyl H-2' chemical shifts with cyclodextrin concentration, treating the racemate as a single compound and fitting the data to equation 5.24. Individual values for the two enantiomers could not be obtained from measurements on the racemate, owing to the failure of MINSQ to successfully carry out non-linear regression analysis of the data for this system. The data obtained for the racemate are illustrated in Figure 5.9, and the parameters obtained are given in Table 5.12.

It can be seen from Figure 5.9 that the variation in chemical shift with cyclodextrin concentration quickly reaches a plateau. This indicates that the complexation is strong. Table 5.12 shows that the formation constant is indeed large. The failure of the model outlined in appendix 1 to produce parameters for the individual enantiomers in this case appeared to be due to the high K_f values rendering the equations insoluble even iteratively.

Table 5.12. Stability and chemical shift data for complex of ±-TA1 with ß-cyclodextrin at pD 7.4, determined from equation 5.24 using MINSQ.

parameter		value	std. dev.
complex formation constant, M^{-1},	K_f	8765	908
shift change on complexation, ppm	D	0.0850	0.0006
correlation coefficient,	r	0.9997	

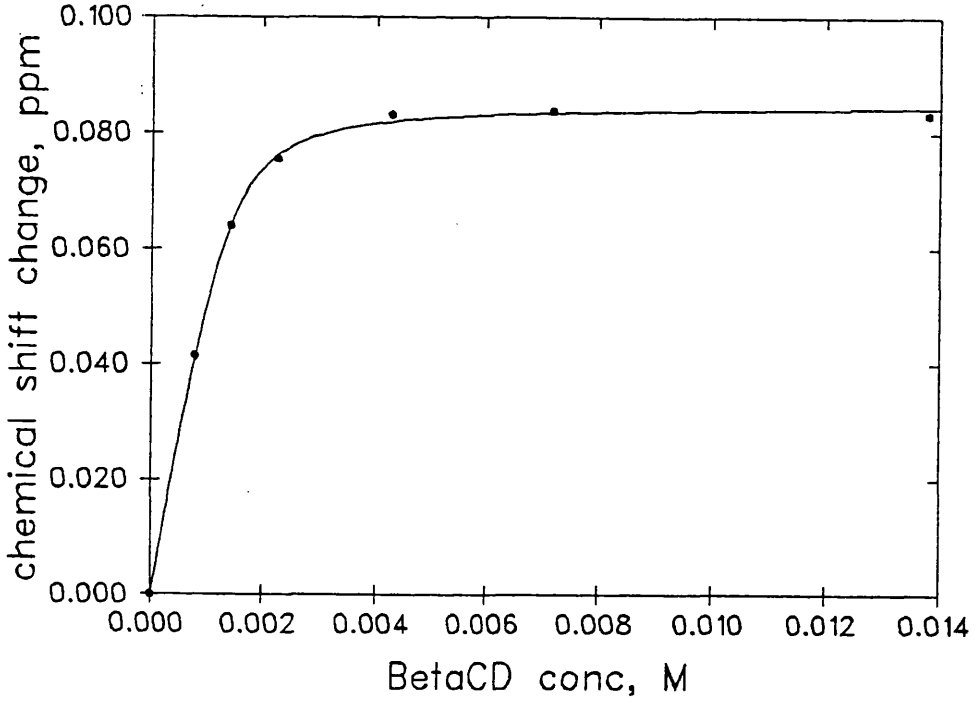


Figure 5.9. Variation in chemical shift of ±TA1 pyridyl H-2 signal with ß-cyclodextrin concentration at pD 7.4 in D2O. [±TA1] = 1.44mM. Data fitted to equation 5.24 using MINSQ.

The degree of enantiotropic splitting in this case also reached a plateau as cyclodextrin concentration was increased. This indicates that the diastereomeric complexes differ significantly in their chemical shifts. No conclusions could be inferred regarding the relative stabilities of the complexes of the two enantiomers from the available data.

5.5.7 ROESY experiment on the ±-TA1-ß-cyclodextrin system.

A 1D-nOe experiment on ±-TA1 (sodium salt)-ß-cyclodextrin (1:1 in D_2O) failed to reveal any enhancements (intra- or inter-molecular). Since the molecular weight of the ß-cyclodextrin-TA1 complex (about 1500) is in the range in which nOe's in the laboratory frame of reference are close to zero, it was decided that a rotating frame (ROESY) experiment might be more appropriate. The result of such an experiment is shown in Figure 5.10.

Intramolecular ROESY cross-peaks (*i.e.* cyclodextrin - cyclodextrin and substrate - substrate) appeared as expected, confirming the previous assignments of signals. Of more interest, however, was the appearance of intermolecular cyclodextrin-substrate ROESY cross-peaks These suggest proximity of cyclodextrin cavity protons (H-3, H-5 and 6-CH_2) to both aromatic moieties (phenol and pyridyl) of ±-TA1. Bearing in mind the 1:1 complex stoichiometry deduced from the Job plot for this system, it seems likely that two different 1:1 complexes may be formed by this compound, one with the pyridyl group included, the other with the phenol group included. Such "bimodal" inclusion has been previously reported. Thus, Kotake and Janzen (1989) reported ESR evidence for the formation of two types of 1:1 complex of an aromatic nitroxide with ß-cyclodextrin.

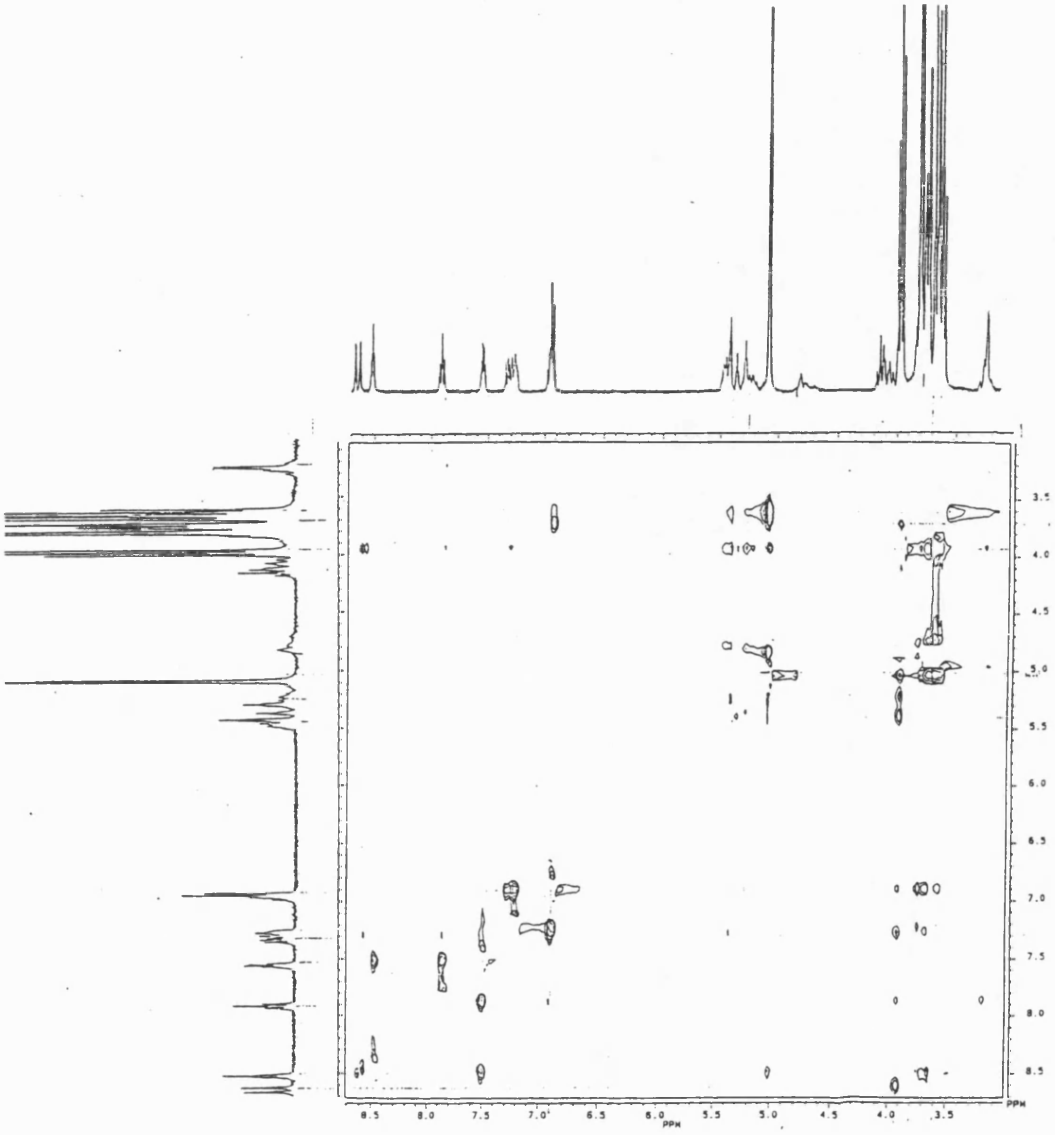


Figure 5.10. 3-9 ppm region of ROESY spectrum of ±-TA1 (3.88mg/ml) as sodium salt with ß-cyclodextrin (11.44mg/ml) in D_2O at 25^{O}C with solvent pre-saturation. The contours represent resonances opposite to the diagonal peak. 1D spectra are also shown.

There is good reason to believe that the observed intermolecular ROESY cross-peaks are due to genuine nuclear Overhauser effects. Other signals can arise in ROESY spectra (Neuhaus and Williamson (1989)), but these could not account for the observed cross-peaks in this case. The spectrum as presented in Figure 5.10 shows only cross-peaks that have a sign opposite to the diagonal peaks. This eliminates the possibility of interference from cross-peaks arising due to HOHAHA transfer, indirect nOe transfer and saturation transfer effects, as these give rise to peaks with the same sign as the diagonal. Coherence transfer (COSY) and false transverse nOe transfer (TOCSY-ROESY) cross-peaks may appear in a spectrum such as Figure 5.10, but these effects only occur between protons in the same spin-spin coupling network. They therefore cannot account for cyclodextrin-substrate cross-peaks.

The symmetry of Figure 5.10 about the diagonal is high, and the general level of noise is low, which suggests that observed cross-peaks are genuine and not artefactual. In order to eliminate the possibility of spurious effects arising due to the saturation of the water signal, the experiment was repeated without solvent suppression. Identical results were obtained.

Chapter 6

Discussion and Conclusions:

Prediction and optimisation of semi-preparative HPLC chiral separations using ß-cyclodextrin-containing eluents

6.1 Introduction

6.1.1 Predictive strategies

With the large number of chiral separation methods now available, it is of importance to be able to rapidly select the best method for a given racemate, particularly since any one approach may only be appropriate for a limited range of compounds. Several approaches to this problem have been devised and applied to the assessment of the applicability of cyclodextrins as resolving agents.

(a) NMR studies

NMR experiments have been used to investigate the chiral discrimination induced by potential chromatographic chiral selectors. Of particular note in this respect is the work of Shah *et al* (1987). Correlation was found between the magnitude of chemical shift changes induced in the NMR spectra of the two enantiomers of a substrate on addition of the chiral (Pirkle-type) selector and the retention order of the enantiomers on a CSP incorporating the same selector. Pirkle and Pochapsky (1987a) observed nuclear Overhauser enhancements between substrate enantiomers and a chiral selector. These indicated that one enantiomer was more closely bound to the selector, and correlated with chromatographic elution order.

Bertucci *et al* (1990) have reported the results of similar experiments involving ß-cyclodextrin and chiral benzodiazepinones. The magnitude of NMR chemical shift changes of substrate protons on addition of cyclodextrin were taken to indicate strength of complexation, and correlation with enantiomeric elution order obtained using Cyclobond I.

Mularz *et al* (1988a) carried out NMR experiments in an attempt to rationalise the fact that pseudoephedrine enantiomers could be resolved using ß-cyclodextrin mobile phases while ephedrine enantiomers could not. Correlations between NMR chiral discrimination and

HPLC enantioselectivity were found in this case.

A variety of other NMR experiments have been used to investigate complex structure, stoichiometry, and stability. These have already been reviewed (Chapter 5), and have obvious application to the rationalisation of chiral discrimination.

(b) Molecular modelling

Computer modelling studies have been widely applied to the investigation of chiral recognition mechanisms, particularly for Pirkle-type chiral selectors, where the analyte-selector interactions are relatively well characterised. Some success has been achieved in rationalising retention order on the basis of computed interaction energies (Taylor, D.R., presented at *2nd Int. Symp. Chiral Sepns,* Guildford, UK (1989)).

Computer modelling has been used to rationalise the retention order of propanolol enantiomers on Cyclobond I (Armstrong, R.D. (1987)). The more retained enantiomer was found to be capable of more effective hydrogen-bonding with the cyclodextrin secondary hydroxyls.

Taylor (1989) has pointed out that the assumptions inherent in current molecular modelling may limit applicability to rationalisation of chiral separations. Most importantly, solvation interactions are ignored. These may play an important part in chiral recognition, particularly in hydrogen-bonding solvents. The time-scale of such computational studies is such that they are unlikely to form the basis for rapid "screening" of analytes for potential chiral recognition on a given phase.

A less involved approach to modelling of complex structures has been used by Mularz (1988). CPK (space-filling) models were used to assess the "fit" of analytes into the ß-cyclodextrin cavity, and some deductions regarding potential enantioselective interactions

made. While far from perfect, several chiral separations were successfully predicted by this rapid approach.

(c) Structural correlations

A widely-used approach to prediction of chiral separations has been to identify structural features necessary in a substrate for chiral recognition by a given technique. A database has recently been devised where reported chiral separations are classified in terms of structural fragments (Roussel, C., presented at *3rd. Int. Symp. Pharm. Biomed. Anal.*, Boston, USA(1991)). This may prove to play an important role in the future selection of chiral chromatographic methods, although the database only covers chiral stationary phases at present.

Pirkle-type CSP's, where the enantioselective interactions are well-defined, have proved the easiest for which to relate structural features with enantioselectivity, and achiral derivatisation is frequently employed to introduce the necessary functionalities. With chiral selectors such as cyclodextrins, however, where the mechanism of chiral recognition is less well-defined, the development of such rules has proved more problematic.

Armstrong and his co-workers have published a number of papers in which structure-selectivity relationships have been investigated. In one such study (Han *et al* (1988)), 43 compounds were investigated. High chiral selectivity was reported for multi-ring compounds in which at least one ring was aromatic, and where the chiral centre was between two aromatic rings or an aromatic ring and a carbonyl group. In a more extensive recent review (Berthod *et al* (1990)), these generalisations were modified. Highest chiral recognition was found in compounds where the chiral centre was part of a ring, or where the chiral centre was attached to at least two sp^2 hybridised carbons. A study of 19 racemic nicotine analogues has also been reported (Seeman *et al* (1988)). In this series, enantioselectivity

was seen to be highly sensitive to small structural changes. The presence of aromatic groups was found to be important in chiral recognition, and fluorine substituents were found to be advantageous.

6.1.2 Optimisation of semi-preparative separations in elution mode

The chromatographic resolution, R_s, of two components of a mixture under "ideal" conditions of low column loading and Gaussian peak shape is related to selectivity, α, retention, k', and column efficiency, N, by equation 6.1 (Hamilton and Sewell (1982)).

$$R_s = 1/4 \, . \, N^{1/2} \, . \, (\alpha - 1) \, (k'/1+k') \qquad \text{(eqn. 6.1).}$$

At high sample loading, where the approximation that all adsorption sites are equivalent no longer holds, peak shapes deviate markedly from Gaussian, retention falls, and resolution is no longer adequately described by equation 6.1. Attempts to develop mathematical models to describe chromatographic behaviour under conditions of column overload have only been partly successful. However, a number of useful generalisations regarding optimisation of preparative separations have been made (McDonald and Bidlingmeyer (1987), Ghodbane and Guiochon (1988, 1988a)), Snyder *et al* (1987, 1989), Guiochon and Katti (1987)).

Selectivity is thought to be the most important factor, the maximisation of which leads to maximised throughput. For example, an increase in α from 1.05 to 1.2 leads to a 15-fold increase in loading for the same resolution (McDonald and Bidlingmeyer (1987)). The optimisation of selectivity under analytical conditions is therefore useful in producing an optimised preparative separation.

The influence of retention is somewhat more complex. It is well established that analytical resolution increases significantly with increasing k', up to a k' of 20, due to the k'/(1+k') term in equation 6.1. However, since a more retained analyte is present at higher relative

concentration on the stationary phase, capacity (*i.e.* loadability) is inversely related to retention. Furthermore, increases in retention at constant loading *per se* result in decreased throughput. Snyder *et al* (1987) have suggested that k' values between 0.5 and 1.5 may be optimal under conditions of column overload. This is somewhat less than that generally considered optimal for analytical resolution. Consequently, direct scale-up of an optimised analytical separation will not necessarily lead to optimised preparative resolution of solutes.

Column efficiency, which determines peak widths, also plays an important part in determining resolution in both analytical and preparative separations. This is largely determined by particle size and column length. The rate at which column efficiency decreases with column loading has been shown to depend on particle size, with larger particles giving a smaller rate of decrease. McDonald and Bidlingmeyer (1987) have therefore argued that the use of long columns packed with large particles gives optimal throughput. This conclusion was supported by Snyder *et al* (1987), from computer simulation data.

Optimisation of semi-preparative separations must take account of all these factors. For resolution of enantiomers, where selectivity is often low compared to that achievable in other separations, it is clear that the maxmisation of selectivity and the use of highly efficient stationary phases will be critical if optically pure materials are to be produced at high throughput.

6.2 Correlations between HPLC and NMR data

It is of value to seek correlations between data produced using ß-cyclodextrin (i) in NMR, (ii) bonded to an HPLC stationary phase, and (iii) as an HPLC eluent additive, because of the potential predictive value of the former techniques with respect to the latter. The NMR experiment employed in these studies occupied only 20 minutes per solute, allowing the rapid screening of racemates for chiral discrimination with ß-cyclodextrin. It is also quicker to carry out initial chromatographic studies on a Cyclobond column rather than using ß-cyclodextrin-containing eluents, because stationary phase type and cyclodextrin concentration (which are important variables in the latter technique) need not be considered using the cyclodextrin bonded-phase. The greater flexibility of the cyclodextrin eluent approach, as well as its greater applicability to preparative separations, may mean that this is the technique of choice for a racemate for which chiral discrimination has been identified.

6.2.1 Observed correlations between available data

The chiral discrimination induced by ß-cyclodextrin achieved in all the HPLC and NMR studies reported herein is summarised in Table 6.1. Literature data is also included where appropriate.

Table 6.1 gives data for 83 racemates. 80 of these were investigated with ß-cyclodextrin-containing eluents, of which 32 were resolved to some degree. 75 were investigated with a Cyclobond I stationary phase, of which 27 exhibited chiral discrimination. NMR data are available for all 83 racemates, of which 53 exhibited chiral discrimination induced by ß-cyclodextrin. Data from all three techniques are available in 72 cases. Of these, 21 exhibited chiral discrimination by all three approaches.

Table 6.1. Summary of enantioselectivity observed using beta-cyclodextrin (a) in HPLC mobile phases (b) in HPLC stationary phase (Cyclobond I) (c) in ^{1}H-NMR (1:1 substrate:cyclodextrin mole ratio)

solute	resolution observed using beta-cyclodextrin in		
	(a) HPLC eluent	(b) HPLC CSP	(c) NMR
tetrahydroisoquinolines			
TQ1	no	no	yes
TQ2	no	no	yes
TQ3	no	no	no
TQ4	no	no	yes
TQ5	no	no	yes
TQ6	no	no	yes
TQ7	no	no	no
phenothiazines			
trimeprazine tartrate	yes	yes	yes
promethazine.HCl	yes	no	yes
methotrimeprazine (tartrate / maleate)	yes	yes	yes
isothipendyl.HCl	no	no	yes
dimethothaizine mesylate	no	no	yes
mandelic acids, etc.			
mandelic acid	yes	no	yes
" " sodium salt	no	no	no
tropic acid		no	no
" " sodium salt	no	no	

Table 6.1 (contd.)

solute	resolution observed using beta-cyclodextrin in		
	(a) HPLC eluent	(b) HPLC CSP	(c) NMR
3-phenyllactic acid	no	no	yes
" " sodium salt	no	no	yes
2-methoxyphenylacetic acid	no	no	yes
O-acetylmandelic acid	no	no	yes
2-chloromandelic acid	no	no	no
4-chloromandelic acid		no	no
" " sodium salt	no	no	
2-methoxymandelic acid		no	no
" " sodium salt	no	no	
3-methoxymandelic acid		no	yes
" " sodium salt	no	no	no
4-methoxymandelic acid	no	no	yes
3-hydroxymandelic acid	no	no	yes
4-hydroxymandelic acid		no	yes
" " sodium salt	no	no	no
2-phenylpropionic acid		no	yes
" " sodium salt	no	no	no
cyclohexylphenylacetic acid	no	yes[1]	yes
methyl mandelate	no	no	yes
ethyl mandelate	no	no	yes
ICI 1 (sodium salt)	no		no
ICI 2 (sodium salt)	no		no
phenylalanine	no	no	no

Table 6.1 (contd.)

solute	resolution observed using beta-cyclodextrin in		
	(a) HPLC eluent	(b) HPLC CSP	(c) NMR
benzoin	yes	yes	yes
thromboxane antagonists (sodium salts)			
TA1	yes	yes	yes
TA2	yes	yes	yes
TA3	yes	yes	yes
TA4	yes	yes	yes
TA5	no	yes	no
TA6	yes	yes	yes
TA7	yes	yes	yes
TA8	no	yes	yes
TA9	yes	yes	yes
TA10	yes	yes	yes
TA11	yes	yes	no
TA12	yes	yes	no
TA13	yes	yes	yes
TA14	yes	yes	yes
TA15	no	no	yes
TA16	no	yes	no
TA17	yes	yes	yes
Other compounds			
pseudo-ephedrine.HCl	yes		yes[2]
ephedrine.HCl	no	no	no[2]

Table 6.1 (contd.)

solute	resolution observed using beta-cyclodextrin in		
	(a) HPLC eluent	(b) HPLC CSP	(c) NMR
norephedrine.HCl	no		no[2]
normetanephrine.HCl	no		no[2]
pheniramine maleate	no	no[3]	yes[3]
chlorpheniramine maleate	yes	yes[3]	yes[3]
brompheniramine maleate	yes	yes[3]	yes[3]
carbinoxamine maleate	yes	yes[3]	yes[3]
doxylamine succinate	no[4]	no[5]	yes[3]
dimethindene maleate	yes	no[3]	yes[3]
mebrophenhydramine.HCl	no	no[3]	yes[3]
neobenodine.HCl	no		no[3]
meclizine.2HCl	no	no[3]	yes[3]
hydroxyzine.HCl	no	no[3]	yes[3]
nomifensine maleate	yes	yes	no[2]
telemzepine.2HCl	no	no	no[2]
methyl phenidate HCl	yes	yes[5]	yes[2]
tryptophan	no		yes[2]
disopyramide phosphate	no	no[5]	yes[2]
propanolol.HCl	yes[4]	yes[1]	yes[6]
metoprolol tartrate	no	no	yes[2]
bupranolol.HCl	no	no	no[2]
bunolol.HCl	no	no	no[2]
chlorthalidone	yes	yes	no[2]
nefopam.HCl	yes	yes	yes[2]

Table 6.1 (contd.)

solute	resolution observed using beta-cyclodextrin in		
	(a) HPLC eluent	(b) HPLC CSP	(c) NMR
tetramisole.HCl	yes	no	yes[2]
oxyphenonium bromide	yes	no	yes[2]
tropicamide	yes	no	no[2]
phenindamine tartrate	yes	no	no[2]
fenoldopam.HCl	no	no	no[2]
prilocaine.HCl	no	no	yes[2]
AM5(6)	no	no	no[2]
verapamil.HCl	no	no	no[2]

Notes: (a) Where information is derived from literature or other sources, this is indicated by superscript numerals, thus [1] = Armstrong (1986); [2] = A.F. Casy, University of Bath, unpublished results; [3] = Mercer (1989); [4] = Mularz (1988); [5] = Han (1988); [6] = Greatbanks (1987).

(b) Carboxylic acid sodium salts were chromatographed at pH 6. Free acids were chromatographed at pH 2. Salts of basic compounds were chromatographed at pH 3.

Of the 32 compounds resolved using ß-cyclodextrin eluents, 27 were also resolved in NMR. Of the 31 compounds resolved using ß-cyclodextrin eluents and for which Cyclobond data are available, 24 were also resolved on Cyclobond I. In only two cases were racemates resolved using ß-cyclodextrin-containing eluents but not by either of the other techniques. It is clear, therefore, that there are strong but not perfect qualitative correlations between the three data sets. If either the NMR technique or the Cyclobond I column had been used to predict which racemates would be resolvable using ß-cyclodextrin eluents, very few potential separations would not have been predicted. If NMR and Cyclobond I data had

been used concurrently to identify potential resolution candidates for the ß-cyclodextrin eluent, only two of the resolutions actually achieved would have been missed.

This conclusion is at first sight very promising with respect to the aim of predicting potential candidates for semi-preparative resolution using ß-cyclodextrin mobile phases. However, a few cautionary points must be made:

(1) The 83 racemates investigated were by no means selected at random. In several cases (particularly the tetrahydroisoquinolines, phenothiazines, and many of the other basic pharmaceuticals), HPLC studies were prompted by the observation of chiral discrimination effects in NMR. In other cases (notably the mandelic acids and thromboxane antagonists), the observed or reported resolution by HPLC of one or more compounds in a series prompted investigation of a series of structurally related compounds by all three techniques.

(2) The NMR and Cyclobond experiments were conducted under non-optimised conditions. Thus, the standard NMR experiment simply involved the addition of a mole equivalent of ß-cyclodextrin to the racemate or its salt in D_2O, with no precise control of pH and no attempt to mimic HPLC conditions (particularly the addition of organic modifiers). The Cyclobond experiments involved the use of a limited range of buffers in acetonitrile-water eluents, with the acetonitrile content reduced from a high level until resolution was observed or retention reached a high level (k' of about 20 was generally the limit used). The use of a wider range of conditions in these experiments may have led to resolution being observed in more cases, at the expense of speed (which, is of course a major requirement for such "predictive" experiments to be of any value).

(3) Only qualititative correlations are being considered. Thus, a racemate that is described as being "resolved" in Table 6.1 may not exhibit high enough selectivity to be useful on a semi-preparative scale. In order to partially counter this objection, literature data reporting

small selectivity values from single enantiomer injections, where resolution of the racemate was not observed, were not included in Table 6.1.

As has been shown for the thromboxane antagonists (section 3.5.3), there are frequently semi-quantitative correlations between the selectivities seen in the two HPLC techniques. There are also sometimes correlations between the magnitude of enantiotropic splitting in NMR and HPLC enantioselectivity. Thus, for the five phenothiazines investigated a statistically significant positive correlation exists between chromatographic selectivity (Table 3.5) and the degree of enantiotropic splitting in the alkyl methyl signal (Table 5.5), although this correlation is far from linear ($r = 0.958$).

Such correlations can only be sought within series of very closely related compounds, in order that chiral discrimination in related NMR signals can be compared. Even within series, such comparisons cannot always be made. For example, in the mandelic acid series, direct comparison of enantiotropic splittings in the spectra of different compounds is meaningless as the enantiotropic splittings occur in different proton signals. Thus, mandelic acid, O-acetylmandelic acid, 3-phenyllactic acid and the mandelate esters exhibit splittings in signals due to the the methine proton at the chiral centre, while other compounds in the series (notably the aryl-substituted analogues) exhibit splittings instead in aromatic proton signals.

6.2.2 Theoretical basis of correlations between NMR chiral discrimination and HPLC enantioselectivity

It is instructive to consider the theoretical basis of the chiral discrimination induced by ß-cyclodextrin in NMR and the two HPLC techniques, in an attempt to rationalise the observed degree of correlation of three data sets.

The theory behind cyclodextrin eluent HPLC was introduced in section 3.1.6. In the

rigourous treatment of Sybilska (1987), which leads to equation 3.11, chromatographic enantioselectivity may arise due to differences in the retentions ($k'_{S.CD}$) and/or the stability constants (K_f) of the diastereomeric complexes. The latter factor leads to separation via the resulting differences in proportions of the complexed forms of the two enantiomers at equilibrium. A number of authors have shown that the latter factor is principally responsible for the observed selectivity in most cases, and have simplified equation 3.11 to equation 3.14. Chromatographic selectivity, α, is then given by

$$\alpha = k'_2/k'_1 = (1 + K_{f1}[CD]_m)/(1 + K_{f2}[CD]_m) \qquad \text{(eqn. 6.2)},$$

which in the limit of infinite cyclodextrin concentration ($[CD]_m$) tends to the ratio of the two stability constants.

The theoretical basis of chiral discrimination in NMR may be derived from the treatment outlined in section 5.1.4 and Appendix 1. The enantiotropic splitting induced in a proton signal by addition of cyclodextrin may be found by subtraction of equations A.17 and A.18. As in the HPLC case discussed above, chiral discrimination may arise due to the differences in stabilities of the diastereomeric complexes (K_{f1} and K_{f2}), and/or due to differences in their intrinsic properties - in this case their chemical shifts (D_1 and D_2). Clearly if, as in HPLC, it is complex stabilities that are the dominant factor, then strong correlations will arise between the two data sets.

A few cases have been investigated in detail, reported either in Chapter 5 or in the literature. In the cases of both ±-TQ1 (Table 5.4) and ±-methyl mandelate (Table 5.7), which are resolved by NMR but not chromatographically, it was shown that the observed NMR chiral discrimination was more likely to have arisen from differences in the intrinsic chemical shifts of the complexes than from differences in their K_f values. Similar findings have been reported by Smith *et al* (1989), who found that the chiral discrimination induced by alpha-cyclodextrin in the ^{19}F-NMR spectrum of racemic phenylalanine derivatives was attributable to differences in intrinsic chemical shifts rather than stabilities of the complexes.

A similar conclusion was reached bu Uekama *et al* (1985) in a ^{1}H-NMR study of the ±-pirprofen-ß-cyclodextrin system.

Even in cases where measurement of intrinsic parameters was not made, there is evidence to suggest that complex stabilities do not contribute significantly to observed chiral discrimination in NMR. Consider the hypothetical example in Figure 6.1, which illustrates the properties to be expected of racemate spectra in the presence of cyclodextrin if complex stability is the major factor responsible for chiral discrimination.

Figure 6.1a shows the spectrum of a hypothetical racemic substrate in the absence of cyclodextrin, consisting of two singlets, at 2.0ppm and 7.0ppm. Figure 6.1b shows the spectrum of the complexed forms of both enantiomers; both complexes exhibiting chemical shifts of 1.0ppm and 9.0ppm. Thus $D_1 = D_2 = +2.0$ppm for the 7.0ppm signal, and $D'_1 = D'_2 = -1.0$ppm for the 1.0ppm signal. If the two enantiomers differ in their complex formation constants such that enantiomer 1 is 60% complexed at equilibrium and enantiomer 2 is 90% complexed at equilibrium on addition of a mole equivalent of ß-cyclodextrin, then the spectrum of the racemate under these conditions will be as shown in Figure 6.1c, with the chemical shifts of the signals due to each enantiomer being determined by a weighted average of the shifts of the complexed and uncomplexed forms in each case, as given by equations A.16 and A.17 (appendix 1). Two important properties of a case where such assumptions apply are illustrated by Figure 6.1c:

(1) The signals due to enantiotropic protons are shifted in the same direction relative to the uncomplexed material, *i.e.* $\Delta\delta_1$ and $\Delta\delta_2$ are of the same sign.

(2) There is a linear relationship between the magnitude of the observed chemical shift change for a given proton $((\Delta\delta_1 + \Delta\delta_2)/2)$ and the magnitude of the enantiotropic splitting observed in that signal $(\Delta\delta_2 - \Delta\delta_1)$

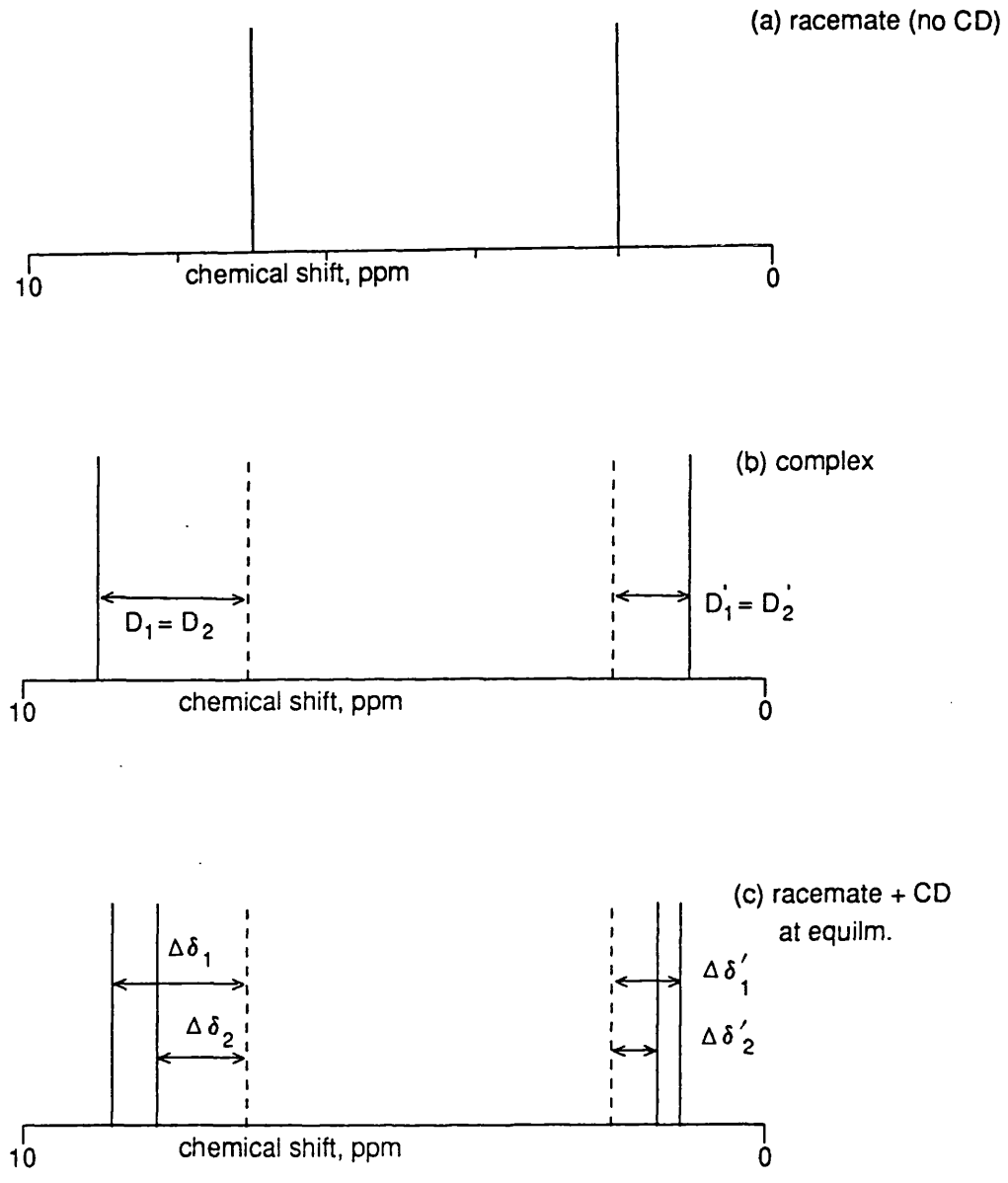


Figure 6.1. Effect of addition of cyclodextrin to a racemate with a two-line NMR spectrum, where the diastereomeric complexes formed differ in their stabilities but not in their intrinsic chemical shifts. For discussion see text.

These properties will be found in racemate-ß-cyclodextrin spectra where differences in complex stabilities are the major factor responsible for chiral discrimination.

Table 6.2 summarises the chemical shift changes in aromatic proton signals of thromboxane antagonists on addition of ß-cyclodextrin. It is apparent that the above properties do not apply to this data. For example, in the case of the phenol H-3 signal of TA1 (and in several other cases), enantiotropic protons are shifted in opposite directions relative to the uncomplexed substrate. For no compound in this series is there a statistically significant correlation found between the chemical shift change and enantiotropic splitting. The fact that differences in complex stability do not appear to influence the NMR spectrum of ±-TA1 with ß-cyclodextrin is notable, since HPLC studies have shown that the differences in complex stabilities in this case must be large, giving rise to a chromatographic selectivity of over 1.6.

Examination of the NMR spectra of all the compounds studied reveals that in none of them are the above criteria satisfied, *i.e.* it appears to be differences in complex chemical shifts that are mainly responsible for enantiotropic splittings in every case.

The fact that differences in complex stabilties, which are known from HPLC to be significant in many cases, do not show up in NMR may be a reflection of the magnitudes of the intrinsic changes in properties involved in the two techniques. In HPLC the capacity factors of the uncomplexed solutes may be quite high (say 10 or above in many cases), while the retention of complexes is nearly zero. Differences in the fractions of the two enantiomers complexed at equilibrium may therefore give rise to comparatively large differences in retention. In NMR, the differences in chemical shift between complexed and uncomplexed forms of a substrate are often small relative to the resolution of the instrument (0.1ppm or less in cases where data is available). Thus, the differences in chemical shifts of the two enantiomers at equilibrium arising from differences in stability constants, being the

Table 6.2 Changes in chemical shifts (ppm) of aromatic proton signals of thromboxane antagonists on addition of beta-cyclodextrin (one mole equiv.). Where chiral discrimination was observed, the chemical shifts of both enantiotropic signals are given. Unresolved signals denoted n.r..

compound	pyridyl signals				aryl signals			
	H-2	H-4	H-5	H-6	H-3	H-4	H-5	H-6
TA1	+0.10, +0.06	-0.02, -0.04	+0.07, +0.06	+0.05, +0.03	+0.01, -0.02	-0.12 (n.r.)	0.0	0.0
TA2	+0.01, -0.05	-0.01, -0.04	+0.07	+0.06, +0.03	-0.04, -0.07	-0.15	+0.04	+0.04
TA3	+0.04, -0.04	+0.01, -0.02	-0.03	+0.10, +0.10	-0.2 (n.r.)	0.0	-0.1 (n.r.)	-0.05
TA4	+0.03	+0.07	+0.03	0.0	-0.07	-0.1 (n.r.)	-0.35 (n.r.)	-0.1 (n.r.)
TA5	+0.16	+0.20	+0.07	+0.06	-0.1 (n.r.)	+0.17	+0.10	+0.07
TA6	+0.20	+0.27, +0.25	+0.30	+0.20	+0.15 (n.r.)	+0.2 (n.r.)	+0.15 (n.r.)	+0.2 (n.r.)
TA13	+0.04, -0.02	-0.03, -0.03	+0.07, +0.06	+0.06, +0.04	n.r.	n.r.	n.r.	n.r.
TA14	+0.11, +0.11	+0.02, +0.02	+0.10	+0.07, +0.05	n.r.	n.r.	n.r.	n.r.
TA15	-0.07 (n.r.)	-0.13, -0.15	+0.05, +0.03	+0.06 (n.r.)	-0.02	0.0	0.0	0.0
TA16	-0.07 (n.r.)	-0.10	0.0	+0.02 (n.r.)	+0.06 (n.r.)	+0.08	+0.06 (n.r.)	0.0
TA17	-0.04, -0.07	-0.03	+0.15, +0.15	+0.13, +0.12	+0.38	+0.08	+0.04	+0.08

difference of two fractions of a small quantity, are generally not observable. Only if the diastereomeric complexes differ in their intrinsic chemical shifts are enantiotropic splittings observable.

While the above argument may be an over-simplification, it would appear to provide rationalisation of the experimental observations. The NMR technique appears to be if anything more sensitive to differences in the structures of the diastereomeric complexes than is HPLC, as evidenced by the larger number of solutes showing chiral discrimination by NMR. The fact that qualitative correlations arise between the two data sets reflects the fact that structural differences (giving rise to chemical shift differences in NMR) are likely to be accompanied by energetic differences (giving rise to HPLC enantioseparation). The incomplete nature of this correlation is a reflection of a lack of direct connection between structural and energetic changes.

6.2.3. Theoretical basis for correlation between Cyclobond I and ß-cyclodextrin eluent data.

The correlation between K_f values and chromatographic enantioselectivity is much less direct in the cyclodextrin bonded-phase approach than is the case with cyclodextrin eluents. A couple of studies involving Cyclobond columns have been reported in which this factor was investigated.

Arnold *et al* (1989) found strong correlations between complex stabilisation energies, derived computationally, and retention times on Cyclobond I within a series of disubstituted benzene isomers. However, strong correlations were not found between different series of compounds. They concluded that non-inclusion effects, particularly the energy barrier for polar substituents to enter the cyclodextrin cavity, remain fairly constant within a series of closely related substrates but vary significantly between series. A similar conclusion was arrived at by Wang *et al* (1990), who compared literature thermodynamic data with retention

times on Cyclobond I, and found little correlation except within series of related compounds.

This lack of correlation probably reflects the fact that the bonding of the cyclodextrin to the silica (*via* the primary hydroxyl groups) to form the Cyclobond phase changes its chemical nature, particularly by hindering interactions with the narrow side of the cavity. Interactions with the "spacer" linking the cyclodextrin to the silica, and with the silica itself, may also contribute to the retention process.

If similar processes are giving rise to enantioselectivity in both HPLC systems, one would anticipate the reversal of elution order of a pair of enantiomers on the Cyclobond I column relative to the ß-cyclodextrin eluent approach. This aspect was not extensively investigated, owing to the non-availability of resolved samples of the enantiomers of most of the compounds studied. In cases where this could be investigated, *e.g.* trimeprazine, brompheniramine, TA1, TA12, elution order was indeed found to be reversed between the two systems. This may not always be the case, however.

The elution order of related racemates was also investigated. Here, reversal of elution order between the two HPLC systems is not necessarily expected. While, retention order may be determined largely by complex stabilities on the Cyclobond column, differences in retention of the uncomplexed solutes also influence retention order when using cyclodextrin-containing eluents. Only in cases (such as the phenothiazine series) where inclusion is known to be strong and thus dominates the retention order in both systems was reversal in order between the systems observed.

It is clear that the predictive value of both Cyclobond HPLC and NMR experiments with respect to cyclodextrin eluent HPLC is limited on theoretical grounds. However, the data presented herein suggests that such experiments may have empirical value.

6.2.4. Practical considerations in predictive experiments

(a) NMR experiments

There are a number of refinements that could be made to the NMR experiments as currently carried out in order to enhance their predictive value with respect to the chromatography. The primary objection to the NMR experiments reported here might be that they were carried out in deuterated water, whereas HPLC solvents contained buffer components and organic modifiers.

The effect of deuterated organic solvents on the chiral discrimination induced in the NMR spectrum of ±-TQ1 is shown in Figure 6.2.

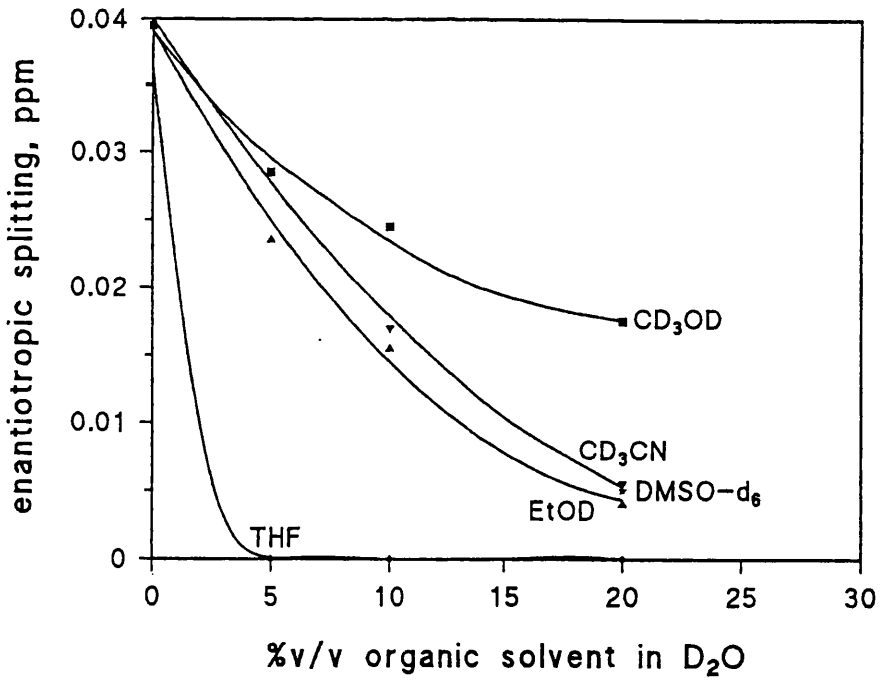


Figure 6.2. Effect of organic modifier on enantiotropic splitting between H-8 proton signals of TQ1 enantiomers on addition of ß-cyclodextrin (one mole equivalent).

The effect of organic modifiers on NMR chiral discrimination in this case was qualitatively similar to that reported in HPLC, *i.e.* a reduction in discrimination with increasing organic modifier content. Attenuation of enantioselectivity was least with methanol, as is generally reported to be the case in HPLC studies.

The use of triethylammonium acetate in NMR experiments was investigated in order to mimic the conditions used in chromatographic studies involving basic compounds . No difference in the spectra of ±-brompheniramine maleate with ß-cyclodextrin with and without these additives (at constant pH) was observed. This is consistent with the observation in chromatographic studies that addition of triethylamine to eluents caused little change in selectivity (although improved peak shapes).

There might be some value in routinely carrying out NMR experiments in mixed aqueous-organic modified solvents. However, a number of considerations arise:
(1) There is no certainty that the effect of organic modifier in the NMR experiment will always be analogous to that in HPLC, since the change of solvent may induce changes in intrinsic chemical shifts of substrates or complexes.
(2) Practically, producing NMR spectra in mixed solvents can be problematic, particularly in ensuring that the spectrometer locks to the deuterated water signal rather than the organic modifer deuterium signal.
(3) If it is assumed that the effect of organic modifier is always to reduce the observed enantioselectivity in both HPLC and NMR, running NMR experiments in mixed solvents will only reduce the number of compounds exhibiting NMR chiral discrimination. Those compounds which are currently resolved by HPLC but not by NMR are even more likely to fall into this category in an experiment modified in this way.

NMR experiments reported herein were carried out without pH control. Since pH has been identified as a major variable in cyclodextrin complexation, it would be more appropriate in

future studies to use buffered solutions. This can be achieved with little practical difficulty.

In a recent paper, Camilleri and Dyke (1990) have reported improved chiral separations on an α_1-acid glycoprotein CSP using D_2O in place of H_2O in mobile phases. Presumably, this effect arises due to differences in hydrogen-bonding properties of the isotopic forms of water. Since hydrogen-bonding is thought to be important in cyclodextrin chiral recognition, it is possible that such differences may also arise with this chiral selector. It might therefore be more appropriate to carry out predictive NMR experiments in H_2O rather than D_2O, using solvent suppression techniques to eliminate the resulting large water peak. Some experiments of this type were carried out. No significant differences in chiral discrimination between the two solvents were observed, and it was not found to be possible to effectively suppress the H_2O peak without inducing severe baseline distortion which on occasions made spectral interpretation difficult. It seems, therefore, that the results may not differ by enough for experiments in non-deuterated water to be worthwhile, bearing in mind the practical difficulties.

The choice of cyclodextrin concentration in the NMR experiments reported herein was not arbitrary, although not necessarily optimal. Chiral discrimination might be expected to increase as cyclodextrin:substrate ratio is increased, due to increasing degree of substrate complexation. However, owing to the limited solubility of ß-cyclodextrin, such an increase could only be achieved by reducing the substrate concentration. This would lead to the need for longer experiment times (*i.e.* a larger number of scans) to maintain signal-to-noise at acceptable levels. The 1:1 mole ratio employed in these studies provided the best balance between degree of complexation and sensitivity. This of course bears little relation to the HPLC situation, where selectivity is independent of substrate concentration at low column loading.

The experiments carried out on the Cyclobond I column were constructed as simply as

possible, involving the use of one or two pH's for each racemate. Control of retention was achieved by varying the acetonitrile content of the eluent. Whilst the use of a wider range of pH's, buffer components and organic modifiers might have led to more racemates being resolved by this technique, the increase in experiment time involved would have diminished the predictive value of the results, since it would have then have been just as rapid to investigate the cyclodextrin-racemate interaction directly using cyclodextrin-containing eluents.

With the development of rapid optimisation strategies for cyclodextrin eluents, it may be that the predictive value of Cyclobond experiments is limited anyway. Since some racemates were better resolved on the Cyclobond I than with ß-cyclodextrin eluents, this approach of course has great value as an analytical technique in its own right.

6.3. NMR determination of complex stability, stoichiometry and structure

6.3.1 Formation constant determination

The determination of cyclodextrin complex formation constants for single compounds by NMR is widely reported in literature, and has been carried out successfully here for (+) and (-) methyl mandelate and for ±-TA1 (treated as a single compound). The experiment is relatively straightforward to carry out, and gives precise results (CV's generally less than 10%). This approach appears to be more facile than the HPLC method (also widely reported). Attempts to measure K_f values by HPLC in this work were not always successful, due mainly to variations in column retentivity during the course of such experiments. Such problems do not arise in the NMR method.

While K_f values determined on single compounds are of great value in inclusion studies, there is no direct connection between strength of complex formation of a racemic compound (*i.e.* the absolute magnitude of K_f values) and the chiral discrimination (*i.e.* the difference between the K_f values of the two enantiomers). Therefore, it would be even more useful to be able to routinely determine the K_f values for two enantiomers from measurements on the racemate, *i.e.* without having pure samples of the two enantiomers. Such data may give a firm guide as to whether chromatographic separation of a pair of enantiomers is likely to be possible, from a relatively rapid series of NMR experiments.

To this end, the theory outlined in Appendix 1 was developed. The resulting equations were found to be circularly dependent, but could be solved iteratively in cases (such as ±-methyl mandelate and ±-TQ1) where the K_f values were small (around 100 M^{-1}), and precise K_f values obtained. However, in strongly complexed cases (such as ±-TA1) solution of the equations was not possible, since the square root of a negative number cropped up during the iteration process. This may have been a function of the iteration method used by the software employed (MINSQ), in which case other software might have more success. As

things stand, the utility of this method is limited by this problem. Routine K_f determination is unlikely to have much predictive value while the method fails for many racemates.

6.3.2 NMR determination of complex stoichiometry and structure

The use of Job's method for determination of complex stoichiometry, and the ROESY technique for investigation of complex structure has been illustrated for ±-TA1. Such experiments are of little predictive value, because of the time involved in carrying them out and because the information they give is not directly related to enantioselectivity of inclusion. Carrying out experiments on resolved enantiomers rather than on racemates would be of greater value in thisrespect. These can only be carried out once samples of enantiomers are available in a given case.

The chief value of NMR experiments of this type may be in rationalising enantioselectivity already demonstrated in NMR or HPLC studies. At present, few examples have been investigated in this way. In the future, the results of NMR experiments on pure enantiomers, coupled with molecular modelling studies, may allow a clearer understanding of the interactions responsible for chiral discrimination, allowing stronger correlations between structure and selectivity to be drawn.

6.3.3 Analytical applications of NMR

As well as the use of the NMR experiment to predict and rationalise HPLC separations, it forms the basis of a useful analytical method in its own right. In several of the cases investigated here, baseline or near-baseline resolution of enantiotropic signals was observed. The observation of baseline resolution is in many cases prevented by the complex mulitiplicity of key proton signals, or by their being obscured by cyclodextrin or solvent resonances.

6.4. Optimisation of semi-preparative separations using cyclodextrin-containing eluents

6.4.1. Summary of semi-preparative separations

The results of the semi-preparative separations reported in Chapter 4 are summarised in able 6.3. Optical purity and throughput were in general reduced as enantioselectivity decreased. However, the results for brompheniramine show that feasible semi-preparative separations can be achieved, even when selectivity is relatively low, by using wider columns and passing partially resolved material through the system a second time.

Table 6.3. Summary of semi-preparative chiral separations carried out using beta-cyclodextrin-containing eluents.

compound		% e.e.		throughput, mg racemate / hr/ g packing material
	α	peak 1	peak 2	
TA1	1.61	99.1	98.4	3.5
trimeprazine	1.33	>98	92	4.4
TA12	1.22	96.3	87.6	2.1
brompheniramine	1.14	95.4	88.0	0.7

Table 6.4 shows the effect of loading on resolution for a wider range of solutes. It is clear from this data that selectivity is crucial in determining loadability, and hence semi-preparative throughput. For slightly more than half of the chiral separations reported in Chapter 3, selectivity (α) was less than 1.2. The approach used to resolve brompheniramine enantiomers semi-preparatively is therefore likely to be the rule rather than the exception.

Table 6.4. Effect of loading on resolution of racemic solutes. Column dimensions: 100 x 4mm in all cases (containing 0.77g packing material). Other conditions vary.

racemate	1 - 10µg on col.			1mg on col.
	k'_1	α	% CRF	% CRF
TA1	10.4	1.61	100	96
trimeprazine	7.0	1.30	99	47
TA3	9.6	1.29	100	86
TA12	20.2	1.22	100	45
TA11	9.1	1.22	100	65
TA7	5.6	1.16	66	36
TA4	7.2	1.14	93	47
TA2	7.3	1.13	81	0
TA10	13.3	1.12	61	0
benzoin	5.3	1.12	50	0

Since chiral separations by this technique are rarely facile, careful optimisation, under analytical conditions to maximise selectivity and at high loading to maximise overall throughput, is important.

6.4.2. Effect of organic modifiers

The choice of organic modifiers in cyclodextrin-containing eluents is influenced by a number of considerations:

(1) Effect on enantioselectivity

It is well established that the presence of organic modifiers in a cyclodextrin-containing

eluent reduces the enantioselectivity by reducing the concentration of available cyclodextrin. For this reason, only low levels (20% v/v or less) of organic modifiers were employed in the eluents used in this work. Methanol is reported to have the lowest affinity for ß-cyclodextrin and therefore gives rise to highest selectivity at a given level fo modifier and cyclodextrin in the eluent. These generalisations are borne out by data for chlorthalidone and benzoin (Table 6.5), and for brompheniramine (Table 4.13).

Table 6.5. Variation in resolution of benzoin and chlorthalidone enantiomers with eluent composition. Column: S5CN 250 x 4.6mm. Loading: 2µg racemate on col.

eluent	[betaCD],	benzoin			chlorthalidone		
	mg/ml	k'_1	α	R_S	k'_1	α	R_S
ACN - water (10:90)	3.3	1.7	1.03	0.5	0.9	1.05	0.5
	6.6	1.3	1.07	0.75	0.8	1.12	0.9
	11.8	0.9	1.08	0.8	0.6	1.19	1.2
	20	0.6	1.10	0.7	0.5	1.26	1.3
ACN - water (5:95)	6.4	1.6	1.11	0.95	1.0	1.24	1.4
	12.8	1.0	1.12	0.95	0.7	1.31	1.5
	20	0.7	1.13	0.85	0.6	1.37	1.5
MeOH - water (10:90)	7.6	2.2	1.13	1.0	1.1	1.28	1.3
	11.0	1.7	1.14	0.9	0.9	1.33	1.5
MeOH - water (5:95)	6.8	4.0	1.15	1.0	1.8	1.31	1.6
	12.0	2.5	1.15	0.9	1.2	1.39	2.6
DMSO - water (5:95)	12.0	1.7	1.13	1.0	1.0	1.27	1.3
	22.0	1.1	1.14	0.9	0.7	1.36	1.5

(2) Effect on cyclodextrin solubility

The variation in ß-cyclodextrin solubility with solvent composition in illustrated in Table 6.6. Methanol was found to markedly reduce the solubility, while the other modifiers caused increased solubility, up to the 20% v/v level investigated.

Table 6.6. Solubility of Beta-cyclodextrin in aqueous-organic solvent mixtures, measured according to the method of Jozwiakowski and Connors (1985) at 20°C. Beta-cyclodextrin concentrations refer to anhydrous material. *Results marked with an asterisk are the mean of 2 determinations.

solvent	betaCD solubility, mg/ml	std. dev.
water	15.7	0.1
acetonitrile - water (5:95, v/v)	20.6	0.2
acetonitrile - water (10:90, v/v)	25.7*	
acetonitrile - water (20:80, v/v)	31.8	1.7
methanol - water (5:95, v/v)	13.0	1.4
methanol - water (10:90, v/v)	11.5*	
methanol - water (20:80, v/v)	7.5*	
acetonitrile - methanol - water (5:5:90, v/v)	15*	
ethanol - water (5:95, v/v)	17.4	0.1
ethanol - water (10:90, v/v)	18.4	0.3
ethanol - water (20:80, v/v)	20.7*	
tetrahydrofuran - water (5:95, v/v)	18.2	0.7
tetrahydrofuran - water (10:90, v/v)	21.9	1.8
tetrahydrofuran - water (20:80, v/v)	26.3*	

While the reasons for this are not clear, this information has important implications in choice of HPLC eluent composition. Table 6.5 illustrates that, while methanol as organic modifier gives highest selectivity at a given cyclodextrin concentration, high selectivity may still be observed using solvents containing DMSO or acetonitrile, since these modifiers allow a higher concentration of cyclodextrin to be added to the mobile phase.

(3) Effect on column efficiency

As has been demonstrated for trimeprazine (Table 4.2), the presence of organic modifier in the eluent improves column efficiency, by "wetting" the hydrophobic alkyl chains on the stationary phase surface and thereby improving mass transfer kinetics. For this reason, studies were not routinely conducted using pure water as eluent. While this would have lead to high selectivity, resolution would have been decreased due to loss of column efficiency.

(4) Effect on retention

Choice of organic modifier was also influenced by the need to optimise retention. For brompheniramine, methanol was preferred as organic modifier because retention values were generally low on the column employed. In the other semi-preparative separations, retention was reduced by the use of acetonitrile in eluents.

6.4.3 Choice of stationary phase

In conventional chromatography, the nature of the stationary phase plays an important part in determining selectivity. In separation of enantiomers using cyclodextrin eluents, however, the role of the stationary phase appears to be small, owing to the low degree of interaction between the cyclodextrin complexes with the stationary phase. For example, Mularz (1988) found that the selectivity between brompheniramine enantiomers was independent of

stationary phase type. This assumption may not hold on very hydrophobic stationary phases, or for derivatised cyclodextrins (Clark (1989), Zukowski and Nowakowski (1989)). In studies reported here, however, stationary phases of low hydrophobicity were mainly used in order to give appropriate retention times with the largely aqueous eluents employed. The role of the stationary phase may therefore be largely limited to providing sufficient retention and efficiency to convert the relatively low selectivity imparted by cyclodextrin-containing eluents into useable resolution.

(a) Optimisation of column efficiency

A number of authors have reported changes in column efficiency on addition of cyclodextrins to HPLC eluents. Reductions of up to 30% were reported by Sybilska (1987). These were attributed either to modifications of the stationary phase by the cyclodextrin, or to mass transfer restrictions due to the large size of cyclodextrin and complex molecules relative to stationary phase pores. This factor was discussed at length by Mularz (1988). A series of wide-pore (300Å) packings were investigated, and found to give impressive resolution of enantiomers in some cases.

Evidence for pore-size effects in the work reported herein was sparse. While many racemates were indeed resolved on a wide-pore column, good resolutions were also obtained on conventional (70 -100Å pore size) stationary phases. It proved impossible to obtain columns of differing pore size but similar retentivity and efficiency, in order that direct comparison could be made.

All the columns employed for separation of enantiomers in these studies were packed with 5µm particles. The use of 3µm particles would have lead to higher innate column efficiency, but placed limitations on column length and/or flow-rate (due to higher back-pressure). The use of larger particles, as suggested by McDonald and Bidlingmeyer (1987), was not

investigated, since it was judged that efficiency would be insufficient to resolve racemates where only low to moderate selectivity was observed. This factor might be worth further study.

(b) Optimisation of retention

The other important factor affecting choice of stationary phase was optimisation of retention. Whereas in conventional chromatography, there are generally no limitations on the range of solvent compositions that can be used to optimise retention, in cyclodextrin chromatography there are fairly strict limitations on organic solvent content of eluents, and it is therefore more critical to select the appropriate stationary phase for use with the optimised mobile phase. For maximum flexibility in optimisation, the availability of a wide range of efficient columns of varying hydrophobicity would be desirable, although this is not always economically possible. The optimisation procedure for a given compound is best started on a column of low hydrophobicity, in order to minimise retention with low levels of eluent organic modifier. More hydrophobic columns can be used subsequently if increased retention is required to improve resolution.

It is difficult to generalise about the optimum k' value for rapid and efficient semi-preparative separations from the data presented herein. For trimeprazine, a fairly high k' (about 10) was found to be optimal, mainly on the grounds that on the short column employed lower retention would have made more critical the appropriate choice of switching times. By reduction of the cyclodextrin content of the eluent, retention was increased giving a greater margin for error in this respect.

For the thromboxane antagonists, and TA12 in particular, the retention times were probably somewhat greater than optimal (k' greater than 10), resulting in sub-optimal throughput. Highest throughput for TA1 was achieved using the highest possible concentration of

ß-cyclodextrin, indicating that further decreases in retention might have been advantageous. However, the use of a less hydrophobic (CN) stationary phase gave retention that was too low for good resolution. Increases in eluent acetonitrile content resulted in markedly lower selectivity. In the absence of a stationary phase of intermediate hydrophobicity between the wide-pore C8 and the cyano phases that were available, the conditions used were about the best that could be achieved.

For brompheniramine, column selection was made mainly on the basis that the CN stationary phase was the only one for which a larger (250 x 10mm) column was available. In the optimisation that was carried out, it was found that low organic modifier and cyclodextrin contents in the eluent, giving maximised retention, were optimal. Ideally, a stationary phase giving somewhat more retention than that achievable on the cyano phase (k' less than 2) might give improved resolution for brompheniramine.

It is clear from the above that pragmatic considerations such as the availability of stationary phases and particular column configurations, and the need for "leeway" in column switching time programming, inevitably involve compromises in throughput.

Another factor affecting column choice is that the recovery system relies on a difference in hydrophobicity between separating and recovery columns. To this end, highly hydrophobic recovery phases were employed. In order to maximise recovery column capacity, the use of a separating column containing as polar a phase as possible, with a suitably "weak" eluent is advantageous. Thus, the recovery column capacities for brompheniramine, which was chromatographed on a cyanopropyl-silica column were significantly higher than those for trimeprazine, which was chromatographed on a octyl-silica phase.

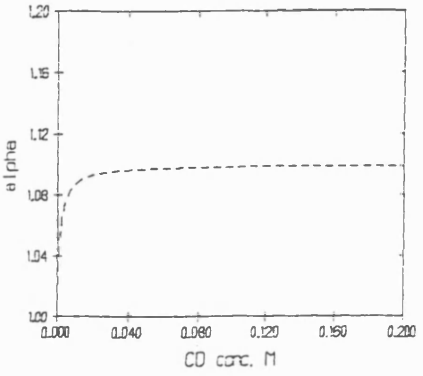
6.4.4. Optimisation of eluent cyclodextrin concentration

In general, it is reported that increases in cyclodextrin concentration in the mobile phase

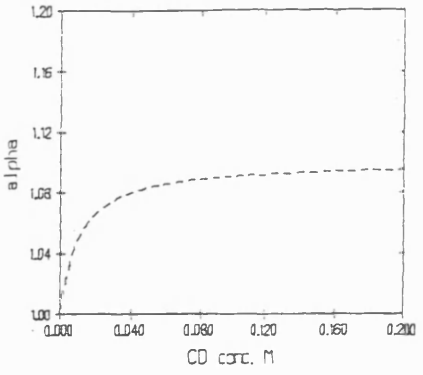
result in improved enantioselectivity (*e.g.* Takeuchi *et al* (1986)). However, for highly complexed solutes the increase in selectivitywith cyclodextrin concentration may reach a plateau before the solubility of the cyclodextrin is reached. Resolution may be optimal at less than the maximum possible cyclodextrin concentration, since retention may be sub-optimal at high cyclodextrin concentration.

The rate of increase of selectivity with cyclodextrin concentration depends on the strength of complexation. This is illustrated in Figure 6.3.

(a)



(b)



(c)

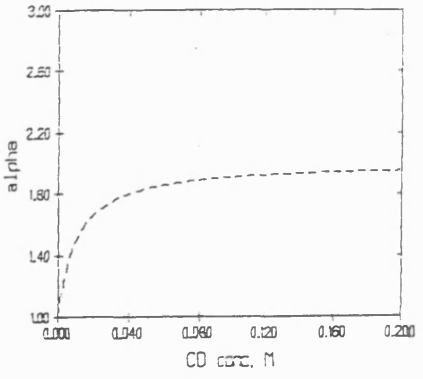


Figure 6.3. Predicted variation of enantioselectivity with cyclodextrin concentration in the absence of organic modifier, simulated using MINSQ from eqn. 6.1.

(a) K_{f1} = 770 M^{-1}; K_{f2} = 700 M^{-1}

(b) K_{f1} = 110 M^{-1}; K_{f2} = 100 M^{-1}

(c) K_{f1} = 200 M^{-1}; K_{f2} = 100 M^{-1}.

N.B.: Beta-cyclodextrin aq. solubility = 0.015M approx. at 20^{o}C.

In Figures 6.3a and b, the selectivity approaches the same value (1.1) as cyclodextrin concentration is increased, as the ratios of the formation constant values are the same. The plateau is reached more quickly in the more strongly complexed case (a). In cases such as (b), the use of a high cyclodextrin concentration is likely to be more critical. In Figure 6.4c, the maximum selectivity is reached at the same rate as in (b), but its value is higher owing to the greater inherent selectivity of the complexation.

These points are further illustrated by the data for chlorthalidone and benzoin. As shown in Table 3.9, benzoin is more strongly complexed than chlorthalidone, although the latter exhibits greater enantioselectivity. The variation of benzoin selectivity with mobile phase composition (Table 6.5) is less marked than that for chlorthalidone, as the selectivity reaches a plateau at a lower cyclodextrin concentration. In a weakly complexed case such as chlorthalidone, the maximisation of cyclodextrin concentration and minimisation of organic modifier content is more likely to be critical than in a more strongly complexed case such as benzoin.

Urea has been reported to dramatically increase the aqueous solubility of ß-cyclodextrin (Pharr *et al* (1989)). This effect was utilised by Hinze *et al* (1989) to achieve improved separations of isomeric benzene derivatives by TLC using ß-cyclodextrin-containing eluents. The addition of 8M urea to the eluent was found to increase ß-cyclodextrin solubility by a factor of 12. Formamide has been used for the same purpose (Collicott, R.J., presented at *2nd. Int. Symp. Chiral Sepns.*, Guildford, UK (1989)).

Urea-containing mobile phases were used to achieve improved separations of some thromboxane antagonists (Table 3.7). In a number of otherwise unresolved cases, partial resolution was observed in the presence of urea (up to 240mg/ml) and increased amounts of ß-cyclodextrin (up to 100mg/ml). Clark (1989) has reported that the use of such eluents gave problems with baseline stability and back-pressure. Such problems were noted in

studies reported herein, but did not prove insurmountable.

The use of urea-containing mobile phases in semi-preparative separations was not investigated, but is a potentially promising approach. The on-line recovery system would probably allow the recovery of resolved enantiomers free from urea as well as the other eluent additives, since urea is highly polar and therefore weakly retained on reversed-phase stationary phases. This approach will only yield benefits for weakly complexed solutes, where the plateau in the selectivity-cyclodextrin concentration relationship is only reached at cyclodextrin concentrations above its solubility in the absence of urea.

6.4.5. Use of triethylamine / base deactivated stationary phases

It is well established (Nahum and Horvath (1981)) that the poor peak shapes observed for basic solutes in RP-HPLC may be attributed to strong (ion-exchange) interactions with acidic residual (unbonded) silanols on the stationary phase. This problem is conventionally overcome by the addition of an excess of a small amine molecule to the eluent, which can effectively compete for these silanol groups. Solute peak asymmetry and retention are thereby reduced (Bij *et al* (1981)).

Mularz (1988) has argued that triethylamine and other amines also improve the kinetics of the cyclodextrin-substrate inclusion process. He showed that markedly improved separations of racemic bases using cyclodextrin-containing eluents could be achieved by use of triethylamine and similar buffers. Berthod *et al* (1990) have suggested that a similar improvement in chromatography on Cyclobond I induced by triethylamine is due to the triethylamine competing with the solute for the cyclodextrin hydroxyl groups. It is not, of course, possible to distinguish the effect of triethylamine on the silanols of a reversed-phase stationary phase from the effect on the inclusion process in the mobile phase.

The addition of triethylamine to eluents has proved to be necessary in work reported here

to obtain good resolution of basic racemates on conventional stationary phases. The recovery of the resolved enantiomers free from the triethylamine, however, proved problematic. This prompted the investigation of base-deactivated stationary phases as a means of achieving good peak shapes for bases without triethylamine. The results obtained for trimeprazine on Hypersil BDS are reported in section 4.2.2. This phase gave very poor results without triethylamine in the eluents, and therefore offered no advantages. Engelhardt and Jungheim (1990) have proposed a test whereby if aniline elutes before phenol a phase may be considered base-deactivated. On the Hypersil BDS phase, phenol was found to elute before aniline.

Zorbax RX-C8, on which aniline was found to elute before phenol, gave more promising results. On this phase, reasonable resolution of a number of basic racemates was achieved without triethylamine in eluents. This material might have some utility in semi-preparative applications. However, even on this column, peak shapes were improved by the addition of triethylamine, indicating that base deactivation was not complete. This improvement occurred whether or not cyclodextrin was present in the eluent. No conclusion could therefore be drawn as to the existence of specific interactions between ß-cyclodextrin and triethylamine.

6.4.6 Effect of eluent pH

As has been demonstrated for the thromboxane antagonists (section 3.5.1), pH can be an important determinant of resolution for those compounds whose pKa falls within the pH range (2 to 7) accessible in HPLC on silica-based stationary phases. For such compounds, optimisation of pH is important. pH effects were found to be smaller for most of the basic compounds studied, since their pKa's are generally above pH 7. Clark (1989) has shown that improved chiral resolution of bases can be achieved at high pH on pH-stable materials such as porous graphitic carbon. The preparative application of this has yet to be

investigated.

6.4.7 Use of ion-pairing agents

Szepesi and Gazdag (1988) found that mobile phases containing cyclodextrins and ion-pairing agents such as sodium dodecylsulphate (achiral) or d-camphorsulphonic acid (chiral) exhibited selectivity for the enantiomers of tobanum, where none was observed in absence of either component. This effect was thought to arise due to enhanced inclusion of ion-pairs formed in the mobile phase.

The use of sodium hexanesulphonate as an alternative to triethylamine in eluents for the resolution of trimeprazine enantiomers has already been described (section 4.2.2). Similar results were obtained for other phenothiazines, and for the use of tetrabutylammonium bromide as ion-pairing agent in the chromatography of mandelic acids, *i.e.* increases in resolution without apparent improvement in selectivity or peak shapes. It seems likely that ion-pairing offers no specific advantage to chiral separations with cyclodextrin-containing mobile phases under the conditions employed here, except as a means of increasing retention. The success reported by Gazdag and Szepesi might be attributable to the higher levels of organic modifier used in their work, solubilising ion-pairs formed in the mobile phase. Such an approach involves compromises in cyclodextrin enantioselectivity, and is therefore unlikely to be widely applicable.

6.4.8 Use of modified cyclodextrins

The use of derivatives of ß-cyclodextrin was investigated on an analytical scale (see section 3.9). The results were distinctly umpromising, with no improvements in selectivity found relative to unmodified ß-cyclodextrin, even at high levels in eluents. This contrasts with the results reported by Clark (1989), and Zukowski and Nowakowski (1989). These additives appear to offer advantages only in "dynamic CSP" mode, where they are adsorbed onto a

hydrophobic stationary phase. The preparative application of such an approach has yet to be investigated, but may have advantages over the use of bonded cyclodextrin columns in terms of flexibility and capacity. Recovery of resolved enantiomers free from the more hydrophobic derivatised cyclodextrins may be more problematic than with the highly polar ß-cyclodextrin itself.

6.4.8 Solute solubility

One of the major limitations on the applicability of ß-cyclodextrin-containing eluents to the semi-preparative separation of enantiomers of pharmaceutical importance is the low solubility of hydrophobic solutes in the largely aqueous eluents employed. In order to separate enantiomers of TA1 (section 4.3), it was necessary to dissolve the racemate in a solvent with a much higher organic content than the mobile phase. This caused some problems with precipitation of cyclodextrin and solute in the injector, which had to be disassembled and cleaned at regular intervals. Attempts to resolved benzoin enantiomers had to be abandoned, owing to the low aqueous solubility (less than 0.1mg/ml) of this solute. The use of very large injections of weak saturated solutions will be the only way of resolving such racemates semi-preparatively.

6.4.9 Recovery of enantiomers following chiral separation

The on-line recovery procedure described in chapter 4 has proved to be effective at recovering resolved enantiomers free from mobile phase components. Careful optimisation was required, however. The most effective procedure was found to be to load the recovery columns to only 50 - 75% of their capacity, allowing the prolonged flushing necessary to remove ß-cyclodextrin and triethylamine. It is therefore important during method development to determine recovery column capacity for the solute in question under the chosen mobile phase conditions.

Of the two recovery phases investigated, the polymeric PRP-1 material was found to have greater capacity than Lichroprep RP18 in all cases. This was presumably due to its greater hydrophobicity. It was thought that the PRP-1 phase, being an aromatic polymer might selectively retain aromatic solutes compared to the non-aromatic cyclodextrin and buffer components (due to pi-pi interactions). There was little evidence for this, however. Other hydrophobic stationary phases might prove useful as recovery columns, and might be investigated in future work.

The feasability of solvent extraction as a recovery procedure was demonstrated for trimeprazine. While this failed to remove triethylamine from the product in this case, the results suggest that solvent extraction would be a viable alternative for acidic or neutral solutes, where no triethylamine is used. Even in these cases, the on-line solid-phase extraction procedure described has several advantages. The resolved enantiomers are concentrated during the recovery procedure, and may be eluted in a volatile solvent. This may be important for thermally labile solutes where the heating needed to evaporate largely aqueous mobile phases would be disadvantageous. The on-line recovery procedure is also ideally suited to automation, with the possibility of overnight operation increasing throughput and saving labour costs.

6.4.10 Semi-preparative separations in displacement mode

The recent publications by Vigh *et al* (1989, 1989a, 1990), demonstrating the possibility of achieving semi-preparative separations on Cyclobond I columns in displacement mode, have brought to the fore the advantages of this mode over elution chromatography in terms of loadability and hence throughput. It seems likely that cyclodextrin-silicas, which have previously not been much used for preparative separations owing to their low capacity, may now find application in this area. The advantage of the cyclodextrin bonded-phase over the cyclodextrin eluents is that recovery of resolved material from the resolving agent is not

necessary, although separation of products from other frequently-used additives such as triethylamine may still be problematic, as has been observed here.

This begs the question as to whether displacement chromatographic separations on achiral stationary phases using cyclodextrin eluents might be possible. If they were, the higher capacity of achiral stationary phases compared to Cyclobond phases might again prove advantageous. Carrying out separations in this mode, however, requires fairly sophisticated instrumentation and considerable method development, and has not been investigated here, or reported in literature using cyclodextrin eluents. Investigation of this approach will clearly be a priority in any continuation of this work.

Appendix 1.

Theoretical basis for the simultaneous determination of the stability constants of the complexes formed by two enantiomers with cyclodextrin using NMR measurements on the racemate.

The theory outlined by Smith *et al* (1989) and described in section 5.1.4. does not hold if more than one entity capable of complex formation is present. since the equilibrium concentration of the host, $[CD_{free}]$, will be reduced by the complexation of the competing entity.

Thus, for enantiomeric guest species S_1 and S_2, and by analogy with equation 5.12, we may define complex stability constants K_{f1} and K_{f2}, given by equations A.1 and A.2:

$$K_{f1} = [S_1.CD]/(([S_1]-[S_1.CD])([CD]-[S_1.CD]-[S_2.CD])) \quad \text{(eqn. A.1)},$$

$$K_{f2} = [S_2.CD]/(([S_2]-[S_2.CD])([CD]-[S_2.CD]-[S_1.CD])) \quad \text{(eqn. A.2)}.$$

By analogy with equation 5.15, the chemical shift changes observed on addition of cyclodextrin will be given by

$$\Delta\delta_1 = ([S_1.CD]/[S_1]).D_1 \quad \text{(eqn. A.3)}$$

and $$\Delta\delta_2 = ([S_2.CD]/[S_2]).D_2 \quad \text{(eqn. A.4)},$$

where $\Delta\delta_1$ and $\Delta\delta_2$ are the observed (equilibrium) chemical shift changes for the enantiotropic proton signals on addition of cyclodextrin concentration [CD] and D_1 and D_2 are the limiting values of $\Delta\delta_1$ and $\Delta\delta_2$ at infinite host concentration.

We may now simplify the algebra as before by defining normalised quantities thus:

$$y_1 = [S_1.CD]/[S_1] = \Delta\delta_1/D_1 \quad \text{(eqn. A.5)},$$

$$y_2 = [S_2.CD]/[S_2] = \Delta\delta_2/D_2 \quad \text{(eqn. A.6)},$$

$$x = [CD]/[S_1] = [CD]/[S_2] \quad \text{(eqn. A.7)},$$

if S_1 and S_2 are in racemic mixture.

$$k_1 = 1/(K_{f1}.[S_1]) \qquad \text{(eqn. A.8),}$$

$$k_2 = 1/(K_{f2}.[S_2]) \qquad \text{(eqn. A.9).}$$

Equations A.1 and A.2 then become

$$k_1 = (x-y_1-y_2)(1-y_1)/y_1 \qquad \text{(eqn A.10)}$$

and $$k_2 = (x-y_1-y_2)(1-y_2)/y_2 \qquad \text{(eqn.A.11)}$$

which may be rearranged to

$$0 = (x-y_2) - y_1(x+k_1-y_2+1) + y_1^2 \qquad \text{(eqn. A.12)}$$

and $$0 = (x-y_1) - y_2(x+k_2-y_1+1) + y_2^2 \qquad \text{(eqn. A.13)}$$

and solved to give

$$y_1 = ((x+k_1-y_2+1) - ((x+k_1-y_2+1)^2 - 4(x-y_2))^{1/2}) / 2 \qquad \text{(eqn. A.14)}$$

and $$y_2 = ((x+k_2-y_1+1) - ((x+k_2-y_1+1)^2 - 4(x-y_1))^{1/2}) / 2 \qquad \text{(eqn. A.15).}$$

Resubstitution then yields

$$\Delta\delta_1 = D_1 . (A_1 - (A_1^2+(\Delta\delta_2/D_2)-([CD]/[S_1]))^{1/2}) \qquad \text{(eqn. A.16)}$$

and $$\Delta\delta_2 = D_2 . (A_2 - (A_2^2+(\Delta\delta_1/D_1)-([CD]/[S_2]))^{1/2}) \qquad \text{(eqn. A.17)}$$

where $$A_1 = (([CD]/[S_1]) + 1/(K_{f1}.[S_1]) - (\Delta\delta_2/D_2) + 1)/2$$

and $$A_2 = (([CD/[S_2]) + 1/(K_{f2}.[S_2]) - (\Delta\delta_1/D_1) + 1)/2$$

and $$[S_1] = [S_2] = 0.5 \text{ x racemate concentration.}$$

It can be seen that equations A.16 resembles equation 5.24, with the introduction of additional $\Delta\delta_2/D_2$ terms reflecting the degree of complexation of the second enantiomer. Similarly, equation A.17 contains $\Delta\delta_1/D_1$ terms to reflect the influence of the first enantiomer.

Equations A.16 and A.17 are thus circularly dependent, and contain four unknowns (D_1, D_2, K_{f1}, and K_{f2}). They are therefore not explicitly soluble, but might be solved iteratively.

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